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(54) Title: MULTIVALENT ANTIGEN-BINDING PROTEINS

(57) Abstract

Compositions of, genetic constructions coding for, and methods for producing multivalent antigen-binding proteins are described and claimed. The methods include purification of compositions containing both monomeric and multivalent forms of single polypeptide chain molecules, and production of multivalent proteins from purified monomers. Production of multivalent proteins may occur by a concentration-dependent association of monomeric proteins, or by rearrangement of regions involving dissociation followed by reassociation of different regions. Bivalent proteins, including homobivalent and heterobivalent proteins, are made in the present invention. Genetic sequences coding for bivalent single-chain antigen-binding proteins are disclosed. Uses include all those appropriate for monoclonal and polyclonal antibodies and fragments thereof, including use as a bispecific antigen-binding molecule.

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Multivalent Antigen-Binding Proteins

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Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial Number 07/796,936, filed Nov. 25, 1991, which is a continuation-in-part of U.S. Patent Application Serial No. 07/512,910 filed April 25, 1990, which is a continuation-in-part of Serial No. 07/299,617, filed Jan. 1, 1989, issued as U.S. Patent No. 4,946,778 (Ladner *et al.*), which was a continuation-in-part of Serial No. 092,110, filed Sept. 2, 1987, and Serial No. 902,971, filed Sept. 2, 1986, now abandoned, the contents of all of which are fully incorporated herein by reference.

Background of the Invention

1. Field of the Invention

The present invention relates generally to the production of antigen-binding molecules. More specifically, the invention relates to multivalent forms of antigen-binding proteins. Compositions of, genetic constructions for, methods of use, and methods for producing these multivalent antigen-binding proteins are disclosed.

2. Description of the Background Art

Antibodies are proteins generated by the immune system to provide a specific molecule capable of complexing with an invading molecule, termed an antigen. Figure 14 shows the structure of a typical antibody molecule. Natural antibodies have two identical antigen-binding sites, both of which are specific to a particular antigen. The antibody molecule "recognizes" the antigen by complexing its antigen-binding sites with areas of the antigen termed epitopes. The epitopes fit into the conformational architecture of the antigen-binding sites of the antibody, enabling the antibody to bind to the antigen.

The antibody molecule is composed of two identical heavy and two identical light polypeptide chains, held together by interchain disulfide bonds (see Fig. 14). The remainder of this discussion will refer only to one light/heavy pair of chains, as each light/heavy pair is identical. Each individual light and heavy chain folds into regions of approximately 110 amino acids, assuming a conserved three-dimensional conformation. The light chain comprises one variable region (termed V_L) and one constant region (C_L), while the heavy chain comprises one variable region (V_H) and three constant regions (C_H1, C_H2 and C_H3). Pairs of regions associate to form discrete structures as shown in Figure 14. In particular, the light and heavy chain variable regions, V_L and V_H,associate to form an "F_V" area which contains the antigen-binding site.

The variable regions of both heavy and light chains show considerable variability in structure and amino acid composition from one antibody molecule to another, whereas the constant regions show little variability. The term "variable" as used in this specification refers to the diverse nature of the amino acid sequences of the antibody heavy and light chain variable regions. Each antibody recognizes and binds antigen through the binding site defined by the association of the heavy and light chain variable regions into an F_V area. The light-chain variable region V_L and the heavy-chain variable region V_H of a particular antibody molecule have specific amino acid sequences that

allow the antigen-binding site to assume a conformation that binds to the antigen epitope recognized by that particular antibody.

Within the variable regions are found regions in which the amino acid sequence is extremely variable from one antibody to another. Three of these so-called "hypervariable" regions or "complementarity-determining regions" (CDR's) are found in each of the light and heavy chains. The three CDR's from a light chain and the three CDR's from a corresponding heavy chain form the antigen-binding site.

Cleavage of the naturally-occurring antibody molecule with the proteolytic enzyme papain generates fragments which retain their antigen-binding site. These fragments, commonly known as Fab's (for Fragment, antigen binding site) are composed of the C_L, V_L, C_H1 and V_H regions of the antibody. In the Fab the light chain and the fragment of the heavy chain are covalently linked by a disulfide linkage.

Recent advances in immunobiology, recombinant DNA technology, and computer science have allowed the creation of single polypeptide chain molecules that bind antigen. These single-chain antigen-binding molecules incorporate a linker polypeptide to bridge the individual variable regions, V_L and V_H, into a single polypeptide chain. A computer-assisted method for linker design is described more particularly in U.S. Patent No. 4,704,692, issued to Ladner *et al.* in November, 1987, and incorporated herein by reference. A description of the theory and production of single-chain antigen-binding proteins is found in U.S. Patent No. 4,946,778 (Ladner *et al.*), issued August 7, 1990, and incorporated herein by reference. The single-chain antigen-binding proteins produced under the process recited in U.S. Patent 4,946,778 have binding specificity and affinity substantially similar to that of the corresponding Fab fragment.

Bifunctional, or bispecific, antibodies have antigen binding sites of different specificities. Bispecific antibodies have been generated to deliver cells, cytotoxins, or drugs to specific sites. An important use has been to deliver host cytotoxic cells, such as natural killer or cytotoxic T cells, to specific cellular targets. (U.D. Staerz, O. Kanagawa, M.J. Bevan, *Nature*

314:628 (1985); S. Songilvilai, P.J. Lachmann, *Clin. Exp. Immunol.* **79:** 315 (1990)). Another important use has been to deliver cytotoxic proteins to specific cellular targets. (V. Raso, T. Griffin, *Cancer Res. 41*:2073 (1981); S. Honda, Y. Ichimori, S. Iwasa, *Cytotechnology 4*:59 (1990)). Another important use has been to deliver anti-cancer non-protein drugs to specific cellular targets (J. Corvalan, W. Smith, V. Gore, *Intl. J. Cancer Suppl. 2*:22 (1988); M. Pimm *et al.*, *British J. of Cancer 61*:508 (1990)). Such bispecific antibodies have been prepared by chemical cross-linking (M. Brennan *et al.*, *Science 229*:81 (1985)), disulfide exchange, or the production of hybrid-hybridomas (quadromas). Quadromas are constructed by fusing hybridomas that secrete two different types of antibodies against two different antigens (Kurokawa, T. *et al.*, *Biotechnology 7*:1163 (1989)).

Summary of the Invention

This invention relates to the discovery that multivalent forms of single-chain antigen-binding proteins have significant utility beyond that of the monovalent single-chain antigen-binding proteins. A multivalent antigen-binding protein has more than one antigen-binding site. Enhanced binding activity, di- and multi-specific binding, and other novel uses of multivalent antigen-binding proteins have been demonstrated or are envisioned here. Accordingly, the invention is directed to multivalent forms of single-chain antigen-binding proteins, compositions of multivalent and single-chain antigen-binding proteins, methods of making and purifying multivalent forms of single-chain antigen-binding proteins, and uses for multivalent forms of single-chain antigen-binding proteins. The invention provides a multivalent antigen-binding protein comprising two or more single-chain protein molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain protein.

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Also provided is a composition comprising a multivalent antigen-binding protein substantially free of single-chain molecules.

Also provided is an aqueous composition comprising an excess of multivalent antigen-binding protein over single-chain molecules.

A method of producing a multivalent antigen-binding protein is provided, comprising the steps of producing a composition comprising multivalent antigen-binding protein and single-chain molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain molecule; separating the multivalent protein from the single-chain molecules; and recovering the multivalent protein.

Also provided is a method of producing multivalent antigen-binding protein, comprising the steps of producing a composition comprising single-chain molecules as previously defined; dissociating the single-chain molecules; reassociating the single-chain molecules; separating the resulting multivalent antigen-binding proteins from the single-chain molecules; and recovering the multivalent proteins.

Also provided is another method of producing a multivalent antigen-binding protein, comprising the step of chemically cross-linking at least two single-chain antigen-binding molecules.

Also provided is another method of producing a multivalent antigen-binding protein, comprising the steps of producing a composition comprising single-chain molecules as previously defined; concentrating said single-chain molecules; separating said multivalent protein from said single-chain molecules; and finally recovering said multivalent protein.

Also provided is another method of producing a multivalent antigen-binding protein comprising two or more single-chain molecules, each single-chain molecule as previously defined, said method comprising: providing a genetic sequence coding for said single-chain molecule; transforming a host

cell or cells with said sequence; expressing said sequence in said host or hosts; and recovering said multivalent protein.

Another aspect of the invention includes a method of detecting an antigen in or suspected of being in a sample, which comprises contacting said sample with the multivalent antigen-binding protein of claim 1 and detecting whether said multivalent antigen-binding protein has bound to said antigen.

Another aspect of the invention includes a method of imaging the internal structure of an animal, comprising administering to said animal an effective amount of a labeled form of the multivalent antigen-binding protein of claim 1 and measuring detectable radiation associated with said animal.

Another aspect of the invention includes a composition comprising an association of a multivalent antigen-binding protein with a therapeutically or diagnostically effective agent.

Another aspect of this invention is a single-chain protein comprising: a first polypeptide comprising the binding portion of the variable region of an antibody light chain; a second polypeptide comprising the binding portion of the variable region of an antibody light chain; a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

Another aspect of the present invention includes the genetic constructions encoding the combinations of regions V_L-V_L and V_H-V_H for single-chain molecules, and encoding multivalent antigen-binding proteins.

Another part of this invention is a multivalent single-chain antigen-binding protein comprising: a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein; a third polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and a peptide linker linking said second and third polypeptides (b) and (d) into said

multivalent protein. Also included are gentic constructions coding for this multivalent single-chain antigen-binding protein.

Also included are replicable cloning or expression vehicles including plasmids, hosts transformed with the aforementioned genetic sequences, and methods of producing multivalent proteins with the sequences, transformed hosts, and expression vehicles.

Methods of use are provided, such as a method of using the multivalent antigen-binding protein to diagnose a medical condition; a method of using the multivalent protein as a carrier to image the specific bodily organs of an animal; a therapeutic method of using the multivalent protein to treat a medical condition; and an immunotherapeutic method of conjugating a multivalent protein with a therapeutically or diagnostically effective agent. Also included are labelled multivalent proteins, improved immunoassays using them, and improved immunoaffinity purifications.

An advantage of using multivalent antigen-binding proteins instead of single-chain antigen-binding molecules or Fab fragments lies in the enhanced binding ability of the multivalent form. Enhanced binding occurs because the multivalent form has more binding sites per molecule. Another advantage of the present invention is the ability to use multivalent antigen-binding proteins as multi-specific binding molecules.

An advantage of using multivalent antigen-binding proteins instead of whole antibodies, is the enhanced clearing of the multivalent antigen-binding proteins from the serum due to their smaller size as compared to whole antibodies which may afford lower background in imaging applications. Multivalent antigen-binding proteins may penetrate solid tumors better than monoclonals, resulting in better tumor-fighting ability. Also, because they are smaller and lack the Fc component of intact antibodies, the multivalent antigen-binding proteins of the present invention may be less immunogenic than whole antibodies. The Fc component of whole antibodies also contains binding sites for liver, spleen and certain other cells and its absence should thus reduce accumulation in non-target tissues.

Another advantage of multivalent antigen-binding proteins is the ease with which they may be produced and engineered, as compared to the myeloma-fusing technique pioneered by Kohler and Milstein that is used to produce whole antibodies.

Brief Description of the Drawings.

The present invention as defined in the claims can be better understood with reference to the text and to the following drawings:

FIG. 1A is a schematic two-dimensional representation of two identical single-chain antigen-binding protein molecules, each comprising a variable light chain region (V_L), a variable heavy chain region (V_H), and a polypeptide linker joining the two regions. The single-chain antigen-binding protein molecules are shown binding antigen in their antigen-binding sites.

FIG. 1B depicts a hypothetical homodivalent antigen-binding protein formed by association of the polypeptide linkers of two monovalent single-chain antigen-binding proteins from Fig. 1A (the Association model). The divalent antigen-binding protein is formed by the concentration-driven association of two identical single-chain antigen-binding protein molecules.

FIG. 1C depicts the hypothetical divalent protein of FIG. 1B with bound antigen molecules occupying both antigen-binding sites.

FIG. 2A depicts the hypothetical homodivalent protein of Figure 1B.

FIG. 2B depicts three single-chain antigen-binding protein molecules associated in a hypothetical trimer.

FIG. 2C depicts a hypothetical tetramer of four single-chain antigen-binding protein molecules.

FIG. 3A depicts two separate and distinct monovalent single-chain antigen-binding proteins, Anti-A single-chain antigen-binding protein and Anti-B single-chain antigen-binding protein, with different antigen specificities, each individually binding either Antigen A or Antigen B.

FIG. **3B** depicts a hypothetical bispecific heterodivalent antigen-binding protein formed from the single-chain antigen-binding proteins of Fig. 3A according to the Association model.

FIG. **3C** depicts the hypothetical heterodivalent antigen-binding protein of FIG. **3B** binding bispecifically, i.e., binding the two different antigens, A and B.

FIG. **4A** depicts two identical single-chain antigen-binding protein molecules, each having a variable light chain region (V_L), a variable heavy chain region (V_H), and a polypeptide linker joining the two regions. The single-chain antigen-binding protein molecules are shown binding identical antigen molecules in their antigen-binding sites.

FIG. **4B** depicts a hypothetical homodivalent protein formed by the rearrangement of the V_L and V_H regions shown in FIG. 4A (the Rearrangement model). Also shown is bound antigen.

FIG. **5A** depicts two single-chain protein molecules, the first having an anti-B V_L and an anti-A V_H, and the second having an anti-A V_L and an anti-B V_H. The figure shows the non-complementary nature of the V_L and V_H regions in each single-chain protein molecule.

FIG. **5B** shows a hypothetical bispecific heterodivalent antigen-binding protein formed by rearrangement of the two single-chain proteins of Figure 5A.

FIG. **5C** depicts the hypothetical heterodivalent antigen-binding protein of FIG. 5B with different antigens A and B occupying their respective antigen-binding sites.

FIG. **6A** is a schematic depiction of a hypothetical trivalent antigen-binding protein according to the Rearrangement model.

FIG. **6B** is a schematic depiction of a hypothetical tetravalent antigen-binding protein according to the Rearrangement model.

FIG. **7** is a chromatogram depicting the separation of CC49/212 antigen-binding protein monomer from dimer on a cation exchange high performance liquid chromatographic column. The column is a PolyCAT A

aspartic acid column (Poly WC, Columbia, MD). Monomer is shown as Peak 1, eluting at 27.32 min., and dimer is shown as Peak 2, eluting at 55.52 min.

FIG. 8 is a chromatogram of the purified monomer from Fig. 7. Monomer elutes at 21.94 min., preceded by dimer (20.135 min.) and trimer (18.640 min.). Gel filtration column, Protein-Pak 300SW (Waters Associates, Milford, MA).

FIG. 9 is a similar chromatogram of purified dimer (20.14 min.) from Fig. 7, run on the gel filtration HPLC column of Fig. 8.

FIG. 10A is an amino acid (SEQ ID NO. 11) and nucleotide (SEQ ID NO. 10) sequence of the single-chain protein comprising the 4-4-20 V_L region connected through the 212 linker polypeptide to the CC49 V_H region.

FIG. 10B is an amino acid (SEQ ID NO. 13) and nucleotide (SEQ ID NO. 12) sequence of the single-chain protein comprising the CC49 V_L region connected through the 212 linker polypeptide to the 4-4-20 V_H region.

FIG. 11 is a chromatogram depicting the separation of the monomer (27.83 min.) and dimer (50.47 min.) forms of the CC49/212 antigen-binding protein by cation exchange, on a PolyCAT A cation exchange column (Poly LC, Columbia, MD).

Fig. 12 shows the separation of monomer (17.65 min.), dimer (15.79 min.), trimer (14.19 min.), and higher oligomers (shoulder at about 13.09 min.) of the B6.2/212 antigen-binding protein. This separation depicts the results of a 24-hour treatment of a 1.0 mg/ml B6.2/212 single-chain antigen-binding protein sample. A TSK G2000SW gel filtration HPLC column was used, Toyo Soda, Tokyo, Japan.

Fig. 13 shows the results of a 24-hour treatment of a 4.0 mg/ml CC49/212 antigen-binding protein sample, generating monomer, dimer, and trimer at 16.91, 14.9, and 13.42 min., respectively. The same TSK gel filtration column was used as in Fig. 12.

Fig. 14 shows a schematic view of the four-chain structure of a human IgG molecule.

Fig. **15A** is an amino acid (SEQ ID NO. 15) and nucleotide (SEQ ID NO. 14) sequence of the 4-4-20/212 single-chain antigen-binding protein with a single cysteine hinge.

Fig. **15B** is an amino acid (SEQ ID NO. 17) and nucleotide (SEQ. ID NO. 16) sequence of the 4-4-20/212 single-chain antigen-binding protein with the two-cysteine hinge.

Fig. **16** shows the amino acid (SEQ ID NO. 19) and nucleotide (SEQ ID NO. 18) sequence of a divalent CC49/212 single-chain antigen-binding protein.

Fig. **17** shows the expression of the divalent CC49/212 single-chain antigen-binding protein of Fig. 16 at 42°C, on an SDS-PAGE gel containing total *E. coli* protein. Lane 1 contains the molecular weight standards. Lane 2 is the uninduced *E. coli* production strain grown at 30°C. Lane 3 is divalent CC49/212 single-chain antigen-binding protein induced by growth at 42°C. The arrow shows the band of expressed divalent CC49/212 single-chain antigen-binding protein.

Fig. **18** is a graphical representation of four competition radioimmunoassays (RIA) in which unlabeled CC49 IgG (open circles) CC49/212 single-chain antigen-binding protein (closed circles) and CC49/212 divalent antigen-binding protein (closed squares) and anti-fluorescein 4-4-20/212 single-chain antigen-binding protein (open squares) competed against a CC49 IgG radiolabeled with ^{125}I for binding to the TAG-72 antigen on a human breast carcinoma extract.

Figure **19A** is an amino acid (SEQ ID NO. 21) and nucleotide (SEQ ID NO. 20) sequence of the single-chain polypeptide comprising the 4-4-20 V_L region connected through the 217 linker polypeptide to the CC49 V_H region.

Figure **19B** is an amino acid (SEQ ID NO. 23) and nucleotide (SEQ ID NO. 22) sequence of the single-chain polypeptide comprising the CC49 V_L region connected through the 217 linker polypeptide to the 4-4-20 V_H region.

Figure **20** is a chromatogram depicting the purification of CC49/4-4-20 heterodimer Fv on a cation exchange high performance liquid chromatographic column. The column is a PolyCAT A aspartic acid column (Poly LC,

Columbia, MD). The heterodimer Fv is shown as peak 5, eluting at 30.10 min.

Figure **21** is a coomassie-blue stained 4-20% SDS-PAGE gel showing the proteins separated in Figure 20. Lane 1 contains the molecular weight standards. Lane 3 contains the starting material before separation. Lanes 4-8 contain fractions 2, 3, 5, 6 and 7 respectively. Lane 9 contains purified CC49/212.

Figure **22A** is a chromatogram used to determine the molecular size of fraction 2 from Figure **20**. A TSK G3000SW gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **22B** is a chromatogram used to determine the molecular size of fraction 5 from Figure **20**. A TSK G3000SW gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **22C** is a chromatogram used to determine the molecular size of fraction 6 from Figure **20**. A TSK G30005W gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **23** shows a Scatchard analysis of the fluorescein binding affinity of the CC49 4-4-20 heterodimer Fv (fraction 5 in Figure **20**).

Figure **24** is a graphical representation of three competition enzyme-linked immunosorbent assays (ELISA) in which unlabeled CC49 4-4-20 Fv (closed squares) CC49/212 single-chain Fv (open squares) and MOPC-21 IgG (+) competed against a biotin-labeled CC49 IgG for binding to the TAG-72 antigen on a human breast carcinoma extract. MOPC-21 is a control antibody that does not bind to TAG-72 antigen.

Figure **25** shows a coomassie-blue stained non-reducing 4-20% SDS-PAGE gel. Lanes 1 and 9 contain the molecular weight standards. Lane 3 contains the 4-4-20/212 CPPC single-chain antigen-binding protein after purification. Lane 4, 5 and 6 contain the 4-4-20/212 CPPC single-chain antigen-binding protein after treatment with DTT and air oxidation. Lane 7 contains 4-4-20/212 single-chain antigen-binding protein.

Figure **26** shows a coomassie-blue stained reducing 4-20% SDS-PAGE gel (samples were treated with β-mercaptoethanol prior to being loaded on the

gel). Lanes 1 and 8 contain the molecular weight standards. Lane 3 contains the 4-4-20/212 CPPC single-chain antigen-binding protein after treatment with *bis*-maleimidehexane. Lane 5 contains peak 1 of *bis*-maleimidehexane treated 4-4-20/212 CPCC single-chain antigen-binding protein. Lane 6 contains peak 3 of *bis*-maleimidehexane treated 4-4-20/212 CPPC single-chain antigen-binding protein.

Detailed Description of the Preferred Embodiments

This invention relates to the discovery that multivalent forms of single-chain antigen-binding proteins have significant utility beyond that of the monovalent single-chain antigen-binding proteins. A multivalent antigen-binding protein has more than one antigen-binding site. For the purposes of this application, "valent" refers to the numerosity of antigen binding sites. Thus, a bivalent protein refers to a protein with two binding sites. Enhanced binding activity, bi- and multi-specific binding, and other novel uses of multivalent antigen-binding proteins have been demonstrated or are envisioned here. Accordingly, the invention is directed to multivalent forms of single-chain antigen-binding proteins, compositions of multivalent and single-chain antigen-binding proteins, methods of making and purifying multivalent forms of single-chain antigen-binding proteins, and new and improved uses for multivalent forms of single-chain antigen-binding proteins. The invention provides a multivalent antigen-binding protein comprising two or more single-chain protein molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain protein.

The term "multivalent" means any assemblage, covalently or non-covalently joined, of two or more single-chain proteins, the assemblage having more than one antigen-binding site. The single-chain proteins composing the

assemblage may have antigen-binding activity, or they may lack antigen-binding activity individually but be capable of assembly into active multivalent antigen-binding proteins. The term "multivalent" encompasses bivalent, trivalent, tetravalent, etc. It is envisioned that multivalent forms above bivalent may be useful for certain applications.

A preferred form of the multivalent antigen-binding protein comprises bivalent proteins, including heterobivalent and homobivalent forms. The term "bivalent" means an assemblage of single-chain proteins associated with each other to form two antigen-binding sites. The term "heterobivalent" indicates multivalent antigen-binding proteins that are bispecific molecules capable of binding to two different antigenic determinants. Therefore, heterobivalent proteins have two antigen-binding sites that have different binding specificities. The term "homobivalent" indicates that the two binding sites are for the same antigenic determinant.

The terms "single-chain molecule" or "single-chain protein" are used interchangeably here. They are structurally defined as comprising the binding portion of a first polypeptide from the variable region of an antibody, associated with the binding portion of a second polypeptide from the variable region of an antibody, the two polypeptides being joined by a peptide linker linking the first and second polypeptides into a single polypeptide chain. The single polypeptide chain thus comprises a pair of variable regions connected by a polypeptide linker. The regions may associate to form a functional antigen-binding site, as in the case wherein the regions comprise a light-chain and a heavy-chain variable region pair with appropriately paired complementarity determining regions (CDRs). In this case, the single-chain protein is referred to as a "single-chain antigen-binding protein" or "single-chain antigen-binding molecule."

Alternatively, the variable regions may have unnaturally paired CDRs or may both be derived from the same kind of antibody chain, either heavy or light, in which case the resulting single-chain molecule may not display a functional antigen-binding site. The single-chain antigen-binding protein

molecule is more fully described in U.S. Patent No. 4,946,778 (Ladner *et al.*), and incorporated herein by reference.

Without being bound by any particular theory, the inventors speculate on several models which can equally explain the phenomenon of multivalence. The inventors' models are presented herein for the purpose of illustration only, and are not to be construed as limitations upon the scope of the invention. The invention is useful and operable regardless of the precise mechanism of multivalence.

Figure 1 depicts the first hypothetical model for the creation of a multivalent protein, the "Association" model. Fig. 1A shows two monovalent single-chain antigen-binding proteins, each composed of a V_L, a V_H, and a linker polypeptide covalently bridging the two. Each monovalent single-chain antigen-binding protein is depicted having an identical antigen-binding site containing antigen. Figure 1B shows the simple association of the two single-chain antigen-binding proteins to create the bivalent form of the multivalent protein. It is hypothesized that simple hydrophobic forces between the monovalent proteins are responsible for their association in this manner. The origin of the multivalent proteins may be traceable to their concentration dependence. The monovalent units retain their original association between the V_H and V_L regions. Figure 1C shows the newly-formed homobivalent protein binding two identical antigen molecules simultaneously. Homobivalent antigen-binding proteins are necessarily monospecific for antigen.

Homovalent proteins are depicted in Figs. 2A through 2C formed according to the Association model. Fig. 1A depicts a homobivalent protein, Fig. 2B a trivalent protein, and Fig. 2C a tetravalent protein. Of course, the limitations of two-dimensional images of three-dimensional objects must be taken into account. Thus, the actual spatial arrangement of multivalent proteins can be expected to vary somewhat from these figures.

A heterobivalent antigen-binding protein has two different binding sites, the sites having different binding specificities. Figures 3A through C depict the Association model pathway to the creation of a heterobivalent protein. Figure 3A shows two monovalent single-chain antigen-binding proteins, Anti-

A single-chain antigen-binding protein and Anti-B single-chain antigen-binding protein, with antigen types A and B occupying the respective binding sites. Figure 3B depicts the heterobivalent protein formed by the simple association of the original monovalent proteins. Figure 3C shows the heterobivalent protein having bound antigens A and B into the antigen-binding sites. Figure 3C therefore shows the heterobivalent protein binding in a bispecific manner.

An alternative model for the formation of multivalent antigen-binding proteins is shown in Figures 4 through 6. This "Rearrangement" model hypothesizes the dissociation of the variable region interface by contact with dissociating agents such as guanidine hydrochloride, urea, or alcohols such as ethanol, either alone or in combination. Combinations and relevant concentration ranges of dissociating agents are recited in the discussion concerning dissociating agents, and in Example 2. Subsequent re-association of dissociated regions allows variable region recombination differing from the starting single-chain proteins, as depicted in Fig. 4B. The homobivalent antigen-binding protein of Figure 4B is formed from the parent single-chain antigen-binding proteins shown in Figure 4A, the recombined bivalent protein having V_L and V_H from the parent monovalent single-chain proteins. The homobivalent protein of Figure 4B is a fully functional monospecific bivalent protein, shown actively binding two antigen molecules.

Figures 5A-5C show the formation of heterobivalent antigen-binding proteins via the Rearrangement model. Figure 5A shows a pair of single-chain proteins, each having a V_L with complementarity determining regions (CDRs) that do not match those of the associated V_H. These single-chain proteins have reduced or no ability to bind antigen because of the mixed nature of their antigen-binding sites, and thus are made specifically to be assembled into multivalent proteins through this route. Figure 5B shows the heterobivalent antigen-binding protein formed whereby the V_H and V_L regions of the parent proteins are shared between the separate halves of the heterobivalent protein. Figure 5C shows the binding of two different antigen molecules to the resultant functional bispecific heterobivalent protein. The Rearrangement model also explains the generation of multivalent proteins of

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a higher order than bivalent, as it can be appreciated that more than a pair of single-chain proteins can be reassembled in this manner. These are depicted in Figures 6A and 6B.

One of the major utilities of the multivalent antigen-binding protein is in the heterobivalent form, in which one specificity is for one type of hapten or antigen, and the second specificity is for a second type of hapten or antigen. A multivalent molecule having two distinct binding specificities has many potential uses. For instance, one antigen binding site may be specific for a cell-surface epitope of a target cell, such as a tumor cell or other undesirable cell. The other antigen-binding site may be specific for a cell-surface epitope of an effector cell, such as the CD3 protein of a cytotoxic T-cell. In this way, the heterobivalent antigen-binding protein may guide a cytotoxic cell to a particular class of cells that are to be preferentially attacked.

Other uses of heterobivalent antigen-binding proteins are the specific targeting and destruction of blood clots by a bispecific molecule with specificity for tissue plasminogen activator (tPA) and fibrin; the specific targeting of pro-drug activating enzymes to tumor cells by a bispecific molecule with specificity for tumor cells and enzyme; and specific targeting of cytotoxic proteins to tumor cells by a bispecific molecule with specificity for tumor cells and a cytotoxic protein. This list is illustrative only, and any use for which a multivalent specificity is appropriate comes within the scope of this invention.

The invention also extends to uses for the multivalent antigen-binding proteins in purification and biosensors. Affinity purification is made possible by affixing the multivalent antigen-binding protein to a support, with the antigen-binding sites exposed to and in contact with the ligand molecule to be separated, and thus purified. Biosensors generate a detectable signal upon binding of a specific antigen to an antigen-binding molecule, with subsequent processing of the signal. Multivalent antigen-binding proteins, when used as the antigen-binding molecule in biosensors, may change conformation upon binding, thus generating a signal that may be detected.

Essentially all of the uses for which monoclonal or polyclonal antibodies, or fragments thereof, have been envisioned by the prior art, can be addressed by the multivalent proteins of the present invention. These uses include detectably-labelled forms of the multivalent protein. Types of labels are well-known to those of ordinary skill in the art. They include radiolabelling, chemiluminescent labeling, fluorochromic labelling, and chromophoric labeling. Other uses include imaging the internal structure of an animal (including a human) by administering an effective amount of a labelled form of the multivalent protein and measuring detectable radiation associated with the animal. They also include improved immunoassays, including sandwich immunoassay, competitive immunoassay, and other immunoassays wherein the labelled antibody can be replaced by the multivalent antigen-binding protein of this invention.

A first preferred method of producing multivalent antigen-binding proteins involves separating the multivalent proteins from a production composition that comprises both multivalent and single-chain proteins, as represented in Example 1. The method comprises producing a composition of multivalent and single-chain proteins, separating the multivalent proteins from the single-chain proteins, and recovering the multivalent proteins.

A second preferred method of producing multivalent antigen-binding proteins comprises the steps of producing single-chain protein molecules, dissociating said single-chain molecules, reassociating the single-chain molecules such that a significant fraction of the resulting composition includes multivalent forms of the single-chain antigen-binding proteins, separating multivalent antigen-binding proteins from single-chain molecules, and recovering the multivalent proteins. This process is illustrated with more detail in Example 2. For the purposes of this method, the term "producing a composition comprising single-chain molecules" may indicate the actual production of these molecules. The term may also include procuring them from whatever commercial or institutional source makes them available. Use of the term "producing single-chain proteins" means production of single-chain proteins by any process, but preferably according to the process set forth in

U.S. Patent No. 4,946,778 (Ladner *et al.*). Briefly, that patent pertains to a single polypeptide chain antigen-binding molecule which has binding specificity and affinity substantially similar to the binding specificity and affinity of the aggregate light and heavy chain variable regions of an antibody, to genetic sequences coding therefore, and to recombinant DNA methods of producing such molecules, and uses for such molecules. The single-chain protein produced by the Ladner *et al.* methodology comprises two regions linked by a linker polypeptide. The two regions are termed the V_H and V_L regions, each region comprising one half of a functional antigen-binding site.

The term "dissociating said single-chain molecules" means to cause the physical separation of the two variable regions of the single-chain protein without causing denaturation of the variable regions.

"Dissociating agents" are defined herein to include all agents capable of dissociating the variable regions, as defined above. In the context of this invention, the term includes the well-known agents alcohol (including ethanol), guanidine hydrochloride (GuHCl), and urea. Others will be apparent to those of ordinary skill in the art, including detergents and similar agents capable of interrupting the interactions that maintain protein conformation. In the preferred embodiment, a combination of GuHCl and ethanol (EtOH) is used as the dissociating agent. A preferred range for ethanol and GuHCl is from 0 to 50% EtOH, vol/vol, 0 to 2.0 moles per liter (M) GuHCl. A more preferred range is from 10-30% EtOH and 0.5-1.0 M GuHCl, and a most preferred range is 20% EtOH, 0.5 M GuHCl. A preferred dissociation buffer contains 0.5 M guanidine hydrochloride, 20% ethanol, 0.05 M TRIS, and 0.01 M $CaCl_2$, pH 8.0.

Use of the term "re-associating said single-chain molecules" is meant to describe the reassociation of the variable regions by contacting them with a buffer solution that allows reassociation. Such a buffer is preferably used in the present invention and is characterized as being composed of 0.04 M MOPS, 0.10 M calcium acetate, pH 7.5. Other buffers allowing the reassociation of the V_L and V_H regions are well within the expertise of one of ordinary skill in the art.

The separation of the multivalent protein from the single-chain molecules occurs by use of standard techniques known in the art, particularly including cation exchange or gel filtration chromatography.

Cation exchange chromatography is the general liquid chromatographic technique of ion-exchange chromatography utilizing anion columns well-known to those of ordinary skill in the art. In this invention, the cations exchanged are the single-chain and multivalent protein molecules. Since multivalent proteins will have some multiple of the net charge of the single-chain molecule, the multivalent proteins are retained more strongly and are thus separated from the single-chain molecules. The preferred cationic exchanger of the present invention is a polyaspartic acid column, as shown in Figure 7. Figure 7 depicts the separation of single-chain protein (Peak 1, 27.32 min.) from bivalent protein (Peak 2, 55.54 min.) Those of ordinary skill in the art will realize that the invention is not limited to any particular type of chromatography column, so long as it is capable of separating the two forms of protein molecules.

Gel filtration chromatography is the use of a gel-like material to separate proteins on the basis of their molecular weight. A "gel" is a matrix of water and a polymer, such as agarose or polymerized acrylamide. The present invention encompasses the use of gel filtration HPLC (high performance liquid chromatography), as will be appreciated by one of ordinary skill in the art. Figure 8 is a chromatogram depicting the use of a Waters Associates' Protein-Pak 300 SW gel filtration column to separate monovalent single-chain protein from multivalent protein, including the monomer (21.940 min.), bivalent protein (20.135 min.), and trivalent protein (18.640 min.).

Recovering the multivalent antigen-binding proteins is accomplished by standard collection procedures well known in the chemical and biochemical arts. In the context of the present invention recovering the multivalent protein preferably comprises collection of eluate fractions containing the peak of interest from either the cation exchange column, or the gel filtration HPLC column. Manual and automated fraction collection are well-known to one of

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ordinary skill in the art. Subsequent processing may involve lyophilization of the eluate to produce a stable solid, or further purification.

A third preferred method of producing multivalent antigen-binding proteins is to start with purified single-chain proteins at a lower concentration, and then increase the concentration until some significant fraction of multivalent proteins is formed. The multivalent proteins are then separated and recovered. The concentrations conducive to formation of multivalent proteins in this manner are from about 0.5 milligram per milliliter (mg/ml) to the concentration at which precipitates begin to form.

The use of the term "substantially free" when used to describe a composition of multivalent and single-chain antigen-binding protein molecules means the lack of a significant peak corresponding to the single-chain molecule, when the composition is analyzed by cation exchange chromatography, as disclosed in Example 1 or by gel filtration chromatography as disclosed in Example 2.

By use of the term "aqueous composition" is meant any composition of single-chain molecules and multivalent proteins including a portion of water. In the same context, the phrase "an excess of multivalent antigen-binding protein over single-chain molecules" indicates that the composition comprises more than 50% of multivalent antigen-binding protein.

The use of the term "cross-linking" refers to chemical means by which one can produce multivalent antigen-binding proteins from monovalent single-chain protein molecules. For example, the incorporation of a cross-linkable sulfhydryl chemical group as a cysteine residue in the single-chain proteins allows cross-linking by mild reduction of the sulfhydryl group. Both monospecific and multispecific multivalent proteins can be produced from single-chain proteins by cross-linking the free cysteine groups from two or more single-chain proteins, causing a covalent chemical linkage to form between the individual proteins. Free cysteines have been engineered into the C-terminal portion of the 4-4-20/212 single-chain antigen-binding protein, as discussed in Example 5 and Example 8. These free cysteines may then be cross-linked to form multivalent antigen-binding proteins.

The invention also comprises single-chain proteins, comprising: (a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain; (b) a second polypeptide comprising the binding portion of the variable region of an antibody light chain; and (c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein. A similar single-chain protein comprising the heavy chain variable regions is also a part of this invention. Genetic sequences encoding these molecules are also included in the scope of this invention. Since these proteins are comprised of two similar variable regions, they do not necessarily have any antigen-binding capability.

The invention also includes a DNA sequence encoding a bispecific bivalent antigen-binding protein. Example 4 and Example 7 discusses in detail the sequences that appear in Figs. 10A and 10B that allow one of ordinary skill to construct a heterobivalent antigen-binding molecule. Figure 10A is an amino acid and nucleotide sequence listing of the single-chain protein comprising the 4-4-20 V_L region connected through the 212 linker polypeptide to the CC49 V_H region. Figure 10B is a similar listing of the single-chain protein comprising the CC49 V_L region connected through the 212 linker polypeptide to the 4-4-20 V_H region. Subjecting a composition including these single-chain molecules to dissociating and subsequent re-associating conditions results in the production of a bivalent protein with two different binding specificities.

Synthesis of DNA sequences is well know in the art, and possible through at least two routes. First, it is well-known that DNA sequences may be synthesized through the use of automated DNA synthesizers *de novo*, once the primary sequence information is known. Alternatively, it is possible to obtain a DNA sequence coding for a multivalent single-chain antigen-binding protein by removing the stop codons from the end of a gene encoding a single-chain antigen-binding protein, and then inserting a linker and a gene encoding a second single-chain antigen-binding protein. Example 6 demonstrates the construction of a DNA sequence coding for a bivalent single-chain antigen-binding protein. Other methods of genetically constructing multivalent single-

chain antigen-binding proteins come within the spirit and scope of the present invention.

Having now generally described this invention the same will better be understood by reference to certain specific examples which are included for purposes of illustration and are not intended to limit it unless otherwise specified.

Example 1

Production of Multivalent Antigen-Binding Proteins During Purification

In the production of multivalent antigen-binding proteins, the same recombinant *E. coli* production system that was used for prior single-chain antigen-binding protein production was used. See Bird, *et al.*, *Science* **242:**423 (1988). This production system produced between 2 and 20% of the total *E. coli* protein as antigen-binding protein. For protein recovery, the frozen cell paste from three 10-liter fermentations (600-900 g) was thawed overnight at 4°C and gently resuspended at 4°C in 50 mM Tris-Hcl, 1.0 mM EDTA, 100 mM KCl, 0.1 mM PMSF, pH 8.0 (lysis buffer), using 10 liters of lysis buffer for every kilogram of wet cell paste. When thoroughly resuspended, the chilled mixture was passed three times through a Manton-Gaulin cell homogenizer to totally lyse the cells. Because the cell homogenizer raised the temperature of the cell lysate to 25 ±5°C, the cell lysate was cooled to 5±2°C with a Lauda/Brinkman chilling coil after each pass. Complete lysis was verified by visual inspection under a microscope.

The cell lysate was centrifuged at 24,300g for 30 min. at 6°C using a Sorvall RC-5B centrifuge. The pellet containing the insoluble antigen-binding protein was retained, and the supernatant was discarded. The pellet was washed by gently scraping it from the centrifuge bottles and resuspending it in 5 liters of lysis buffer/kg of wet cell paste. The resulting 3.0- to 4.5-liter suspension was again centrifuged at 24,300g for 30 min at 6°C, and the

supernatant was discarded. This washing of the pellet removes soluble *E. coli* proteins and can be repeated as many as five times. At any time during this washing procedure the material can be stored as a frozen pellet at -20°C. A substantial time saving in the washing steps can be accomplished by utilizing a Pellicon tangential flow apparatus equipped with 0.22-μm microporous filters, in place of centrifugation.

The washed pellet was solubilized at 4°C in freshly prepared 6 M guanidine hydrochloride, 50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, pH 8.0 (dissociating buffer), using 9 ml/g of pellet. If necessary, a few quick pulses from a Heat Systems Ultrasonics tissue homogenizer can be used to complete the solubilization. The resulting suspension was centrifuged at 24,300g for 45 min at 6°C and the pellet was discarded. The optical density of the supernatant was determined at 280 nm and if the OD_{280} was above 30, additional dissociating buffer was added to obtain an OD_{280} of approximately 25.

The supernatant was slowly diluted into cold (4-7°C) refolding buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, pH 8.0) until a 1:10 dilution was reached (final volume 10-20 liters). Re-folding occurs over approximately eighteen hours under these conditions. The best results are obtained when the GuHCl extract is slowly added to the refolding buffer over a 2-h period, with gentle mixing. The solution was left undisturbed for at least a 20-h period, and 95% ethanol was added to this solution such that the final ethanol concentration was approximately 20%. This solution was left undisturbed until the flocculated material settled to the bottom, usually not less than sixty minutes. The solution was filtered through a 0.2 um Millipore Millipak 200. This filtration step may be optionally preceded by a centrifugation step. The filtrate was concentrated to 1 to 2 liters using an Amicon spiral cartridge with a 10,000 MWCO cartridge, again at 4°C.

The concentrated crude antigen-binding protein sample was dialyzed against Buffer A (60 mM MOPS, 0.5 mM Ca acetate, pH 6.0-6.4) until the conductivity was lowered to that of Buffer A. The sample was then loaded on a 21.5 x 250-mm polyaspartic acid PolyCAT A column, manufactured by Poly

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LC of Columbia, Maryland. If more than 60 mg of protein is loaded on this column, the resolution begins to deteriorate; thus, the concentrated crude sample often must be divided into several PolyCAT A runs. Most antigen-binding proteins have an extinction coefficient of about 2.0 ml mg^{-1} cm^{-1} at 280 nm and this can be used to determine protein concentration. The antigen-binding protein sample was eluted from the PolyCAT A column with a 50-min linear gradient from Buffer A to Buffer B (see Table 1). Most of the single-chain proteins elute between 20 and 26 minutes when this gradient is used. This corresponds to an eluting solvent composition of approximately 70% Buffer A and 30% Buffer B. Most of the bivalent antigen-binding proteins elute later than 45 minutes, which correspond to over 90% Buffer B.

Figure 7 is a chromatogram depicting the separation of single-chain protein from bivalent CC49/212 protein, using the cation-exchange method just described. Peak 1, 27.32 minutes, represents the monomeric single-chain fraction. Peak 2, 55.52 minutes, represents the bivalent protein fraction.

Figure 8 is a chromatogram of the purified monomeric single-chain antigen-binding protein CC49/212 (Fraction 7 from Fig. 7) run on a Waters Protein-Pak 300SW gel filtration column. Monomer, with minor contaminates of dimer and trimer, is shown. Figure 9 is a chromatogram of the purified bivalent antigen-binding protein CC49/212 (Fraction 15 from Fig. 7) run on the same Waters Protein-Pak 300SW gel filtration column as used in Fig. 8.

cation exchange and gel filtration chromatography, can be used to separate the single-chain protein monomer from the multivalent antigen-binding proteins. In the first method, monomeric and multivalent antigen-binding proteins were separated by using cation exchange HPLC chromography, using a polyaspartate column (PolyCAT A). This was a similar procedure to that used in the final purification of the antigen-binding proteins as described in Example 1. The load buffer was 0.06 M MOPS, 0.001 M Calcium Acetate pH 6.4. In the second method, the monomeric and multivalent antigen-binding proteins were separated by gel filtration HPLC chromatography using as a load buffer 0.04 M MOPS, 0.10 M Calcium Acetate pH 7.5. Gel filtration chromatography separates proteins based on their molecular size.

Once the antigen-binding protein sample was loaded on the cation exchange HPLC column, a linear gradient was run between the load buffer (0.04 to 0.06 M MOPS, 0.000 to 0.001 M calcium acetate, 0 to 10% glycerol pH 6.0-6.4) and a second buffer (0.04 to 0.06 M MOPS, 0.01 to 0.02 M calcium acetate, 0 to 10% glycerol pH 7.5). It was important to have extensively dialyze the antigen-binding protein sample before loading it on the column. Normally, the conductivity of the sample is monitored against the dialysis buffer. Dialysis is continued until the conductivity drops below 600 μS. Figure 11 shows the separation of the monomeric (27.83 min.) and bivalent (50.47 min.) forms of the CC49/212 antigen-binding protein by cation exchange. The chromatographic conditions for this separation were as follows: PolyCAT A column, 200 x 4.6mm, operated at 0.62 ml/min.; load buffer and second buffer as in Example 1; gradient program from 100 percent load buffer A to 0 percent load buffer A over 48 mins; sample was CC49/212, 1.66 mg/ml; injection volume 0.2 ml. Fractions were collected from the two peaks from a similar chromatogram and identified as monomeric and bivalent proteins using gel filtration HPLC chromatography as described below.

Gel filtration HPLC chromatography (TSK G2000SW column from Toyo Soda, Tokyo, Japan) was used to identify and separate monomeric single-chain and multivalent antigen-binding proteins. This procedure has been described by Fukano, *et al., J. Chromatography* 166:47 (1978).

Multimerization (creation of multivalent protein from monomeric single-chain protein) was by treatment with 0.5 M GuHCl and 20% EtOH for the times indicated in Table 2A followed by dialysis into the chromatography buffer. Figure 12 shows the separation of monomeric (17.65 min.), bivalent (15.79 min.), trivalent (14.19 min.), and higher oligomers (shoulder at about 13.09 min.) of the B6.2/212 antigen-binding protein. The B6.2/212 single-chain antigen-binding protein is described in Colcher, D., *et al.*, *J. Nat. Cancer Inst. 82*:1191-1197 (1990)). This separation depicts the results of a 24-hour multimerization treatment of a 1.0 mg/ml B6.2/212 antigen-binding protein sample. The HPLC buffer used was 0.04 M MOPS, 0.10 M calcium acetate, 0.04% sodium azide, pH 7.5.

Figure 13 shows the results of a 24-hour treatment of a 4.0 mg/ml CC49/212 antigen-binding protein sample, generating monomeric, bivalent and trivalent proteins at 16.91, 14.9, and 13.42 min., respectively. The HPLC buffer was 40 mM MOPS, 100 mM calcium acetate, pH 7.35. Multimerization treatment was for the times indicated in Table 2.

The results of Example 2A are shown in Table 2A. Table 2A shows the percentage of bivalent and other multivalent forms before and after treatment with 20% ethanol and 0.5M GuHCl. Unless otherwise indicated, percentages were determined using a automatic data integration software package.

Table 2A
Summary of the generation of bivalent and higher multivalent forms of B6.2/212 and CC49/212 proteins using guanidine hydrochloride and ethanol

protein	Time (hours)	Concentration (mg/ml)	% monomer	% dimer	% trimer	% multimers
CC49/212	0	0.25	86.7	11.6	1.7	0.0
	0	1.0[2]	84.0	10.6	5.5	0.0
	0	4.0	70.0	17.1	12.9[1]	0.0
	2	0.25[2]	62.9	33.2	4.2	0.0
	2	1.0	24.2	70.6	5.1	0.0
	2	4.0	9.3	81.3	9.5	0.0
	26	0.25	16.0	77.6	6.4	0.0
	26	1.0	9.2	82.8	7.9	0.0
	26	4.0	3.7	78.2	18.1	0.0
B6.2/212	0	0.25	100.0	0.0	0.0	0.0
	0	1.0	100.0	0.0	0.0	0.0
	0	4.0	100.0	0.0	0.0	0.0
	2	0.25[2]	98.1	1.9	0.0	0.0
	2	1.0	100.0	0.0	0.0	0.0
	2	4.0	90.0	5.5	1.0	0.0
	24	0.25	45.6	37.5	10.2	6.7
	24	1.0	50.8	21.4	12.3	15.0
	24	4.0	5.9	37.2	25.7	29.9

[1] Based on cut out peaks that were weighted.
[2] Average of two experiments.

B. Process Using Urea and Ethanol

Multivalent antigen-binding proteins were produced from purified single-chain proteins in the following way. First the purified single-chain protein at a concentration of 0.25-1 mg/ml was dialyzed against 2M urea, 20% ethanol (EtOH), and 50mM Tris buffer pH 8.0, for the times indicated in Table 2B. This combination of dissociating agents is thought to disrupt the V_L/V_H interface, allowing the V_H of a first single-chain molecule to come into contact with a V_L from a second single-chain molecule. Other dissociating agents such as isopropanol or methanol should be substitutable for EtOH.

Following the initial dialysis, the protein was dialyzed against the load buffer for the final HPLC purification step.

Gel filtration HPLC chromatography (TSK G2000SW column from Toyo Soda, Tokyo, Japan) was used to identify and separate monomeric single-chain and multivalent antigen-binding proteins. This procedure has been described by Fukano, *et al.*, *J. Chromatography* **166**:47 (1978).

The results of Example 2B are shown in Table 2B. Table 2B shows the percentage of bivalent and other multivalent forms before and after treatment with 20% ethanol and urea. Percentages were determined using an automatic data integration software package.

Table 2B

Summary of the generation of bivalent and higher multivalent forms of B6.2/212 and CC49/212 proteins using urea and ethanol

protein	Time (hours)	Concentration (mg/ml)	% monomer	% dimer	% trimer	% multimers
B6.2	0	0.25	44.1	37.6	15.9	2.4
	0	1.0	37.7	33.7	19.4	9.4
	3	0.25	22.2	66.5	11.3	0.0
	3	1.0	13.7	69.9	16.4	0.0

Example 3

Determination of Binding Constants

Three anti-fluorescein single-chain antigen-binding proteins have been constructed based on the anti-fluorescein monoclonal antibody 4-4-20. The three 4-4-20 single-chain antigen-binding proteins differ in the polypeptide linker connecting the V_H and V_L regions of the protein. The three linkers used were 202', 212 and 216 (see Table 3). Bivalent and higher forms of the 4-4-20 antigen-binding protein were produced by concentrating the purified monomeric single-chain antigen-binding protein in the cation exchange load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) to 5 mg/ml. The

bivalent and monomeric forms of the 4-4-20 antigen-binding proteins were separated by cation exchange HPLC (polyaspartate column) using a 50 min. linear gradient between the load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) and a second buffer (0.06 M MOPS, 0.02 M calcium acetate pH 7.5). Two 0.02 ml samples were separated, and fractions of the bivalent and monomeric protein peaks were collected on each run. The amount of protein contained in each fraction was determined from the absorbance at 278 nm from the first separation. Before collecting the fractions from the second separation run, each fraction tube had a sufficient quantity of 1.03×10^{5} M fluorescein added to it, such that after the fractions were collected a 1-to-1 molar ratio of protein-to-fluorescein existed. Addition of fluorescein stabilized the bivalent form of the 4-4-20 antigen-binding proteins. These samples were kept at 2°C (on ice).

The fluorescein dissociation rates were determined for each of these samples following the procedures described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe,* E.W. Voss, Ed., CRC Press, Boca Raton, FL (1984). A sample was first diluted with 20 mM HEPES buffer pH 8.0 to 5.0×10^{-8} M 4-4-20 antigen-binding protein. 560 μl of the 5.0×10^{-8} M 4-4-20 antigen-binding protein sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at 2°C and the fluorescence was read. 140 μl of 1.02×10^{-5} M fluoresceinamine was added to the cuvette, and the fluorescence was read every 1 minute for up to 25 minutes (see Table 4).

The binding constants (K_a) for the 4-4-20 single-chain antigen-binding protein monomers diluted in 20 mM HEPES buffer pH 8.0 in the absence of fluorescein were also determined (see Table 4).

The three polypeptide linkers in these experiments differ in length. The 202', 212 and 216 linkers are 12, 14 and 18 residues long, respectively. These experiments show that there are two effects of linker length on the 4-4-20 antigen-binding proteins: first, the shorter the linker length the higher the fraction of bivalent protein formed; second, the fluorescein dissociation rates of the monomeric single-chain antigen-binding proteins are effected more by the linker length than are the dissociation rates of the bivalent antigen-binding

proteins. With the shorter linkers 202′ and 212, the bivalent antigen-binding proteins have slower dissociation rates than the monomers. Thus, the linkers providing optimum production and binding affinities for monomeric and bivalent antigen-binding proteins may be different. Longer linkers may be more suitable for monomeric single-chain antigen-binding proteins, and shorter linkers may be more suitable for multivalent antigen-binding proteins.

Table 3

Linker Designs

V_L	Linker	V_H	Linker Name	Reference
-KLEIE	GKSSGSGSESKS[1]	TQKLD-	202′	Bird *et al.*
-KLEIK	GSTSGSGKSSEGKG[2]	EVKLD-	212	Bedzyk *et al.*
-KLEIK	GSTSGSGKSSEGSGSTKG[3]	EVKLD-	216	This application
-KLVLK	GSTSGKPSEGKG[4]	EVKLD-	217	This application

(1) SEQ ID NO. 1
(2) SEQ ID NO. 2
(3) SEQ ID NO. 3
(4) SEQ ID NO. 4

Table 4

Effects of Linkers on the SCA Protein Monomers and Dimers

	Linker		
	202′	212	216
Monomer Fraction Ka Dissociation rate	 0.47 $0.5 \times 10^9\ M^{-1}$ $8.2 \times 10^{-3}\ s^{-1}$	 0.66 $1.0 \times 10^9\ M^{-1}$ $4.9 \times 10^{-3}\ s^{-1}$	 0.90 $1.3 \times 10^9\ M^{-1}$ $3.3 \times 10^{-3}\ s^{-1}$
Dimer Fraction Dissociation rate	 0.53 $4.6 \times 10^{-3}\ s^{-1}$	 0.34 $3.5 \times 10^{-3}\ s^{-1}$	 0.10 $3.5 \times 10^{-3}\ s^{-1}$
Monomer/Dimer Dissociation rate ratio	 1.8	 1.4	 0.9

Example 4

bivalent and monomeric forms of the 4-4-20 antigen-binding proteins were separated by cation exchange HPLC (polyaspartate column) using a 50 min. linear gradient between the load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) and a second buffer (0.06 M MOPS, 0.02 M calcium acetate pH 7.5). Two 0.02 ml samples were separated, and fractions of the bivalent and monomeric protein peaks were collected on each run. The amount of protein contained in each fraction was determined from the absorbance at 278 nm from the first separation. Before collecting the fractions from the second separation run, each fraction tube had a sufficient quantity of 1.03×10^{5} M fluorescein added to it, such that after the fractions were collected a 1-to-1 molar ratio of protein-to-fluorescein existed. Addition of fluorescein stabilized the bivalent form of the 4-4-20 antigen-binding proteins. These samples were kept at 2°C (on ice).

The fluorescein dissociation rates were determined for each of these samples following the procedures described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe,* E.W. Voss, Ed., CRC Press, Boca Raton, FL (1984). A sample was first diluted with 20 mM HEPES buffer pH 8.0 to 5.0×10^{-8} M 4-4-20 antigen-binding protein. 560 μl of the 5.0×10^{-8} M 4-4-20 antigen-binding protein sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at 2°C and the fluorescence was read. 140 μl of 1.02×10^{-5} M fluoresceinamine was added to the cuvette, and the fluorescence was read every 1 minute for up to 25 minutes (see Table 4).

The binding constants (K_a) for the 4-4-20 single-chain antigen-binding protein monomers diluted in 20 mM HEPES buffer pH 8.0 in the absence of fluorescein were also determined (see Table 4).

The three polypeptide linkers in these experiments differ in length. The 202', 212 and 216 linkers are 12, 14 and 18 residues long, respectively. These experiments show that there are two effects of linker length on the 4-4-20 antigen-binding proteins: first, the shorter the linker length the higher the fraction of bivalent protein formed; second, the fluorescein dissociation rates of the monomeric single-chain antigen-binding proteins are effected more by the linker length than are the dissociation rates of the bivalent antigen-binding

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proteins. With the shorter linkers 202′ and 212, the bivalent antigen-binding proteins have slower dissociation rates than the monomers. Thus, the linkers providing optimum production and binding affinities for monomeric and bivalent antigen-binding proteins may be different. Longer linkers may be more suitable for monomeric single-chain antigen-binding proteins, and shorter linkers may be more suitable for multivalent antigen-binding proteins.

Table 3

Linker Designs

V_L	Linker	V_H	Linker Name	Reference
-KLEIE	GKSSGSGSESKS[1]	TQKLD-	202′	Bird *et al.*
-KLEIK	GSTSGSGKSSEGKG[2]	EVKLD-	212	Bedzyk *et al.*
-KLEIK	GSTSGSGKSSEGSGSTKG[3]	EVKLD-	216	This application
-KLVLK	GSTSGKPSEGKG[4]	EVKLD-	217	This application

(1) SEQ ID NO. 1
(2) SEQ ID NO. 2
(3) SEQ ID NO. 3
(4) SEQ ID NO. 4

Table 4

Effects of Linkers on the SCA Protein Monomers and Dimers

	Linker		
	202′	212	216
Monomer			
Fraction	0.47	0.66	0.90
Ka	$0.5 \times 10^9\ M^{-1}$	$1.0 \times 10^9\ M^{-1}$	$1.3 \times 10^9\ M^{-1}$
Dissociation rate	$8.2 \times 10^{-3}\ s^{-1}$	$4.9 \times 10^{-3}\ s^{-1}$	$3.3 \times 10^{-3}\ s^{-1}$
Dimer			
Fraction	0.53	0.34	0.10
Dissociation rate	$4.6 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$
Monomer/Dimer			
Dissociation rate ratio	1.8	1.4	0.9

Example 4

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Genetic Construction of a Mixed-Fragment Bivalent Antigen-Binding Protein

The genetic constructions for one particular heterobivalent antigen-binding protein according to the Rearrangement model are shown in Figures 10A and 10B. Figure 10A is an amino acid and nucleotide sequence listing of the 4-4-20 V_L/212/CC49 V_H construct, coding for a single-chain protein with a 4-4-20 V_L, linked via a 212 polypeptide linker to a CC49 V_H. Figure 10B is a similar listing showing the CC49 V_L/212/4-4-20 V_H construct, coding for a single-chain protein with a CC49 V_L, linked via a 212 linker to a 4-4-20 V_H. These single-chain proteins may recombine according to the Rearrangement model to generate a heterobivalent protein comprising a CC49 antigen-binding site linked to a 4-4-20 antigen-binding site, as shown in Figure 5B.

"4-4-20 V_L" means the variable region of the light chain of the 4-4-20 mouse monoclonal antibody (Bird, R.E. *et al., Science* **242:**423 (1988)). The number "212" refers to a specific 14-residue polypeptide linker that links the 4-4-20 V_L and the CC49 V_H. See *Bedzyk, W.D.* et al., *J. Biol. Chem. 265:18615-18620 (1990).* "CC49 V_H" is the variable region of the heavy chain of the CC49 antibody, which binds to the TAG-72 antigen. The CC49 antibody was developed at The National Institutes of Health by Schlom, et al. *Generation and Characterization of B72.3 Second Generation Monoclonal Antibodies Reactive With The Tumor-associated Glycoprotein 72 Antigen,* Cancer Research 48:4588-4596 (1988).

Insertion of the sequences shown in FIGS. 10A and 10B, by standard recombinant DNA methodology, into a suitable plasmid vector will enable one of ordinary skill in the art to transform a suitable host for subsequent expression of the single-chain proteins. See Maniatis et al., *Molecular Cloning, A Laboratory Manual*, p. 104, Cold Spring Harbor Laboratory (1982), for general recombinant techniques for accomplishing the aforesaid goals; see also U.S. Patent 4,946,778 (Ladner *et al.*) for a complete

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description of methods of producing single-chain protein molecules by recombinant DNA technology.

To produce multivalent antigen-binding proteins from the two single-chain proteins, 4-4-20V_L-212/CC49V_H and CC49V_L/212/4-4-20V_H, the two single-chain proteins are dialyzed into 0.5 M GuHCl/20% EtOH being combined in a single solution either before or after dialysis. The multivalent proteins are then produced and separated as described in Example 2.

Example 5

Preparation of Multivalent Antigen-Binding Proteins by Chemical Cross-Linking

Free cysteines were engineered into the C-terminal of the 4-4-20/212 single-chain antigen-binding protein, in order to chemically crosslink the protein. The design was based on the hinge region found in antibodies between the C_H1 and C_H2 regions. In order to try to reduce antigenicity in humans, the hinge sequence of the most common IgG class, IgG1, was chosen. The 4-4-20 Fab structure was examined and it was determined that the C-terminal sequence GluH216-ProH217-ArgH218, was part of the C_H1 region and that the hinge between C_H1 and C_H2 starts with ArgH218 or GlyH219 in the mouse 4-4-20 IgG2A antibody. Figure 14 shows the structure of a human IgG. The hinge region is indicated generally. Thus the hinge from human IgG1 would start with LysH218 or SerH219. (See Table 5).

The C-terminal residue in most of the single-chain antigen-binding proteins described to date is the amino acid serine. In the design for the hinge region, the C-terminal serine in the 4-4-20/212 single-chain antigen-binding protein was made the first serine of the hinge and the second residue of the hinge was changed from a cysteine to a serine. This hinge cysteine normally forms a disulfide bridge to the C-terminal cysteine in the light chain.

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TABLE 5

	218
IgG2A mouse[1]	E P R G P T I K P C P P C L C -
IgG1 human[2]	A E P K S C D K T H T C P P C -
SCA[3]	- - V T V S
SCA* Hinge design 1[4]	- - V T V S S D K T H T C
SCA* Hinge design 2[5]	- - V T V S S D K T H T C P P C

* - single-chain antigen-binding protein

(1) SEQ ID NO. 5
(2) SEQ ID NO. 6
(3) SEQ ID NO. 7
(4) SEQ ID NO. 8
(5) SEQ ID NO. 9

There are possible advantages to having two C-terminal cysteines, for they might form an intramolecular disulfide bond, making the protein recovery easier by protecting the sulfurs from oxidation. The hinge regions were added by introduction of a BstE II restriction site in the 3'-terminus of the gene encoding the 4-4-20/212 single-chain antigen-binding protein (see Figures 15A-15B).

The monomeric single-chain antigen-binding protein containing the C-terminal cysteine can be purified using the normal methods of purifying a single-chain antigen-binding proteins, with minor modifications to protect the free sulfhydryls. The cross-linking could be accomplished in one of two ways. First, the purified single-chain antigen-binding protein could be treated with a mild reducing agent, such as dithiothreitol, then allowed to air oxidize to form a disulfide-bond between the individual single-chain antigen-binding proteins. This type of chemistry has been successful in producing heterodimers from whole antibodies (Nisonoff *et al.*, Quantitative Estimation of the Hybridization of Rabbit Antibodies, *Nature 4826*:355-359 (1962); Brennan *et al.*, Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments, *Science 229*:81-83 (1985)). Second, chemical crosslinking agents such as *bis*maleimidehexane could be used to cross-link two single-chain antigen-binding proteins by their C-terminal cysteines. See Partis *et al.*, *J. Prot. Chem.* 2:263-277 (1983).

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Example 6

Genetic Construction of Bivalent Antigen-Binding Proteins

Bivalent antigen-binding proteins can be constructed genetically and subsequently expressed in *E. coli* or other known expression systems. This can be accomplished by genetically removing the stop codons at the end of a gene encoding a monomeric single-chain antigen-binding protein and inserting a linker and a gene encoding a second single-chain antigen-binding protein. We have constructed a gene for a bivalent CC49/212 antigen-binding protein in this manner (see Figure 16). The CC49/212 gene in the starting expression plasmid is in an Aat II to Bam H1 restriction fragment (see Bird *et al.*, Single-Chain Antigen-Binding Proteins, *Science 242:*423-426 (1988); and Whitlow *et al.*, Single-Chain F_V Proteins and Their Fusion Proteins, *Methods* 2:97-105 (1991)). The two stop codons and the Bam H1 site at the C-terminal end of the CC49/212 antigen-binding protein gene were replaced by a single residue linker (Ser) and an Aat II restriction site. The resulting plasmid was cut with Aat II and the purified Aat II to Aat II restriction fragment was ligated into Aat II cut CC49/212 single-chain antigen-binding protein expression plasmid. The resulting bivalent CC49/212 single-chain antigen-binding protein expression plasmid was transfected into an *E. coli* expression host that contained the gene for the cI857 temperature-sensitive repressor. Expression of single-chain antigen-binding protein in this system is induced by raising the temperature from 30°C to 42°C. Fig. 17 shows the expression of the divalent CC49/212 single-chain antigen-binding protein of Fig. 16 at 42°C, on an SDS-PAGE gel containing total *E. coli* protein. Lane 1 contains the molecular weight standards. Lane 2 is the uninduced *E. coli* production strain grown at 30°C. Lane 3 is divalent CC49/212 single-chain antigen-binding protein induced by growth at 42°C. The arrow shows the band of expressed divalent CC49/212 single-chain antigen-binding protein.

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Example 7

Construction, Purification, and Testing of 4-4-20/CC49 Heterodimer F_V With 217 Linkers.

The goals of this experiment were to produce, purify and analyze for activity a new heterodimer Fv that would bind to both fluorescein and the pan-carcinoma antigen TAG-72. The design consisted of two polypeptide chains, which associated to form the active heterodimer Fv. Each polypeptide chain can be described as a mixed single-chain Fv (mixed sFv). The first mixed sFv (GX 8952) comprised a 4-4-20 variable light chain (V_L) and a CC-49 variable heavy chain (V_H) connected by a 217 polypeptide linker (Figure 19A). The second mixed sFv (GX 8953) comprised a CC-49 V_L and a 4-4-20 V_H connected by a 217 polypeptide linker (Figure 19B). The sequence of the 217 polypeptide linker is shown in Table 3. Construction of analogous CC49/4-4-20 heterodimers connected by a 212 polypeptide linker as described in Example 4.

Results

A. Purification

One 10-liter fermentation of each mixed sFv was grown on casein digest-glucose-salts medium at 32°C to an optical density at 600 nm of 15 to 20. The mixed sFv expression was induced by raising the temperature of the fermentation to 42°C for one hour. 277gm (wet cell weight) of *E. coli* strain GX 8952 and 233gm (wet cell weight) of *E. coli* strain GX 8953 were harvested in a centrifuge at 7000g for 10 minutes. The cell pellets were kept and the supernate discarded. The cell pellets were frozen at -20°0C for storage.

2.55 liters of "lysis/wash buffer" (50mM Tris/ 200mM NaCl/ 1 mM EDTA, pH 8.0) was added to both of the mixed sFv's cell pellets, which were previously thawed and combined to give 510gm of total wet cell weight. After complete suspension of the cells they were then passed through a Gaulin homogenizer at 9000psi and 4°C. After this first pass the temperature increased to 23°C. The temperature was immediately brought down to 0°C using dry ice and methanol. The cell suspension was passed through the Gaulin homogenizer a second time and centrifuged at 8000 rpm with a Dupont GS-3 rotor for 60 minutes. The supernatant was discarded after centrifugation and the pellets resuspended in 2.5 liters of "lysis/wash buffer" at 4°C. This suspension was centrifuged for 45 minutes at 8000 rpm with the Dupont GS-3 rotor. The supernatant was again discarded and the pellet weighed. The pellet weight was 136.1 gm.

1300ml of 6M Guanidine Hydrochloride/50mM Tris/50mM KCl/10mM $CaCl_2$ pH 8.0 at 4°C was added to the washed pellet. An overhead mixer was used to speed solubilization. After one hour of mixing, the heterodimer GuHCl extract was centrifuged for 45 minutes at 8000 rpm and the pellet was discarded. The 1425ml of heterodimer Fv 6M GuHCl extract was slowly added (16 ml/min) to 14.1 liters of "Refold Buffer" (50mM Tris/50mM KCl/10mM $CaCl_2$, pH 8.0) under constant mixing at 4°C to give an approximate dilution of 1:10. Refolding took place overnight at 4°C.

After 17 hours of refolding the anti-fluorescein activity was checked by a 40% quenching assay, and the amount of active protein calculated. 150mg total active heterodimer Fv was found by the 40% quench assay, assuming a 54,000 molecular weight.

4 liters of prechilled (4°C) 190 proof ethanol was added to the 15 liters of refolded heterodimer with mixing for 3 hours. The mixture sat overnight at 4°C. A flocculent precipitate had settled to the bottom after this overnight treatment. The nearly clear solution was filtered through a Millipak-200 (0.22μ) filter so as to not disturb the precipitate. A 40% quench assay showed that 10% of the anti-fluorescein activity was recovered in the filtrate.

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The filtered sample of heterodimer was dialyzed, using a Pellicon system containing 10,000 dalton MWCO membranes, with "dialysis buffer" 40mM MOPS/0.5mM Calcium Acetate (CaAc), pH 6.4 at 4°C. 20 liters of dialysis buffer was required before the conductivity of the retentate was equal to that of the dialysis buffer (~500μS). After dialysis the heterodimer sample was filtered through a Millipak-20 filter, O.22μ. After this step a 40% quench assay showed there was 8.8 mg of active protein.

The crude heterodimer sample was loaded on a Poly CAT A cation exchange column at 20ml/min. The column was previously equilibrated with 60mM MOPS, 1 mM CaAc pH 6.4, at 4°C, (Buffer A). After loading, the column was washed with 150ml of "Buffer A" at 15ml/min. A 50min linear gradient was performed at 15ml/min using "Buffer A" and "Buffer B" (60mM MOPS, 20mM CaAc pH 7.5 at 4°C). The gradient conditions are presented in Table 6. "Buffer C" comprises 60mM MOPS, 100mM $CaCl_2$, pH 7.5.

Table 6

Time	%A	%B	%C	Flow
0:00	100.0	0.0	0.0	15ml/min
50:00	0.0	100.0	0.0	15ml/min
52:00	0.0	100.0	0.0	15ml/min
54:00	0.0	0.0	100.0	15ml/min
58:00	0.0	0.0	100.0	15ml/min
60:00	100.0	0.0	0.0	15ml/min

Approximately 50ml fractions were collected and analyzed for activity, purity, and molecular weight by size-exclusion chromatography. The fractions were not collected by peaks, so contamination between peaks is likely. Fractions 3 through 7 were pooled (total volume - 218ml), concentrated to 50ml and dialyzed against 4 liters of 60mM MOPS, 0.5mM CaAc pH 6.4 at 4°C overnight. The dialyzed pool was filtered through a O.22μ filter and

checked for absorbance at 280nm. The filtrate was loaded onto the PolyCAT A column, equilibrated with 60mM MOPS, 1 mM CaAc pH 6.4 at 4°C, at a flow rate of 10ml/min. Buffer B was changed to 60mM MOPS, 10mM CaAc pH 7.5 at 4°C. The gradient was run as in Table 6. The fractions were collected by peak and analyzed for activity, purity, and molecular weight. The chromatogram is shown in Figure 20. Fraction identification and analysis is presented in Table 7.

Table 7

Fraction Analysis of the Heterodimer Fv protein

Fraction No.	A_{280} reading	Total Volume (ml)	HPLC-SE Elution Time (min)
2	0.161	36	20.525
3	0.067	40	
4	0.033	40	
5	0.178	45	19.133
6	0.234	50	19.163
7	0.069	50	
8	0.055	40	

Fractions 2 to 7 and the starting material were analyzed by SDS gel electrophoresis, 4-20%. A picture and description of the gel is presented in Figure 21.

B. HPLC Size Exclusion Results

Fractions 2, 5, and 6 correspond to the three main peaks in Figure 20 and therefore were chosen to be analyzed by HPLC size exclusion. Fraction 2 corresponds to the peak that runs at 21.775 minutes in the preparative purification (Figure 20), and runs on the HPLC sizing column at 20.525 minutes, which is in the monomeric position (Figure 22A). Fractions 5 and 6 (30.1 and 33.455 minutes, respectively, in Figure 20) run on the HPLC sizing column (Figures 22B and 22C) at 19.133 and 19.163 minutes,

respectively (see Table 7). Therefore, both of these peaks could be considered dimers. 40% Quenching assays were performed on all fractions of this purification. Only fraction 5 gave significant activity. 2.4 mg of active CC49 4-4-20 heterodimer Fv was recovered in fraction 5, based on the Scatchard analysis described below.

C. N-terminal sequencing of the fractions

The active heterodimer Fv fraction should contain both polypeptide chains. N-terminal sequence analysis showed that fractions 5 and 6 displayed N-terminal sequences consistent with the prescence of both CC49 and 4-4-20 polypeptides and fraction 2 displayed a single sequence corresponding to the CC49/212/4-4-20 polypeptide only. We believe that fraction 6 was contaminated by fraction 5 (see Figure 20), since only fraction 5 had significant activity.

D. Anti-fluorescein activity by Scatchard analysis

The fluorescein association constants (Ka) were determined for fractions 5 and 6 using the fluorescence quenching assay described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe*, E.W. Voss, ed., CRC Press, Boca Raton, FL (1984). Each sample was diluted to approximately 5.0×10^{-8} M with 20 mM HEPES buffer pH 8.0. 590 μl of the 5.0×10^{-8} M sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at room temperature. In a second cuvette 590 μl of 20 mM HEPES buffer pH 8.0 was added. To each cuvette was added 10 μl of 3.0×10^{-7} M fluorescein in 20 mM HEPES buffer pH 8.0, and the fluorescence recorded. This is repeated until 140 μl of fluorescein had been added. The resulting Scatchard analysis for fraction 5 shows a binding constant of 1.16×10^{9} M^{-1} for fraction #5 (see Figure 23). This is very close to the 4-4-20/212 sFv constant of 1.1×10^{9} M^{-1} (see Pantoliano *et al.*, *Biochemistry 30*:10117-10125 (1991)). The R intercept on the Scatchard analysis represents the fraction of active material. For fraction 5, 61% of the

material was active. The graph of the Scatchard analysis on fraction 6 shows a binding constant of 3.3 x 10^8 M^{-1} and 14% active. The activity that is present in fraction 6 is most likely contaminants from fraction 5.

E. Anti-TAG-72 activity by competition ELISA

The CC49 monoclonal antibody was developed by Dr. Jeffrey Schlom's group, Laboratory of Tumor Immunology and Biology, National Cancer Institute. It binds specifically to the pan-carcinoma tumor antigen TAG-72. See Muraro, R., *et al.*, *Cancer Research 48*:4588-4596 (1988).

To determine the binding properties of the bivalent CC49/4-4-20 Fv (fraction 5) and the CC49/212 sFv, a competition enzyme-linked immunosorbent assay (ELISA) was set up in which a CC49 IgG labeled with biotin was competed against unlabeled CC49/4-4-20 Fv and the CC49/212 sFv for binding to TAG-72 on a human breast carcinoma extract (see Figure 24). The amount of biotin-labeled CC49 IgG was determined using a preformed complex with avidin and biotin coupled to horse radish peroxidase and O-phenylenediamine dihydrochloride (OPD). The reaction was stopped with 4N H_2SO_4 (sulfuric acid), after 10 min. and the optical density read at 490nm. This competition ELISA showed that the bivalent CC49/4-4-20 Fv binds to the TAG-72 antigen. The CC49/4-4-20 Fv needed a two hundred-fold higher protein concentration to displace the IgG than the single-chain Fv.

Example 8

Cross-Linking Antigen-Binding Dimers

We have chemically crosslinked dimers of 4-4-20/212 antigen-binding protein with the two cysteine C-terminal extension (4-4-20/212 CPPC single-chain antigen-binding protein) in two ways. In Example 5 we describe the design and genetic construction of the 4-4-20/212 CPPC single-chain antigen-binding protein (hinge design 2 in Table 5). Figure 15B shows the nucleic

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acid and protein sequences of this protein. After purifying the 4-4-20/212 CPPC single-chain antigen-binding protein, using the methods described in Whitlow and Filpula, *Meth. Enzymol. 2*:97 (1991), dimers were formed by two methods. First, the free cysteines were mildly reduced with dithiothreitol (DTT) and then the disulfide-bonds between the two molecules were allowed to form by air oxidation. Second, the chemical crosslinker *bis*-maleimidehexane was used to produce dimers by crosslinking the free cysteines from two 4-4-20/212 CPPC single-chain antigen-binding proteins.

A 0.1 mg/ml solution of the 4-4-20/212 CPPC single-chain antigen-binding protein was mildly reduced using 1 mM DTT, 50 mM HEPES, 50mM NaCl, 1 mM EDTA buffer pH 8.0 at 4°C. The samples were dialyzed against 50mM HEPES, 50 mM NaCl, 1 mM EDTA buffer pH 8.0 at 4°C overnight, to allow the oxidation of free sulfhydrals to intermolecular disulfide-bonds. Figure 25 shows a non-reducing SDS-PAGE gel after the air oxidation; it shows that approximately 10% of the 4-4-20/212 CPPC protein formed dimers with molecular weights around 55,000 Daltons.

A 0.1 mg/ml solution of the 4-4-20/212 CPPC single-chain antigen-binding protein was treated with 2 mM *bis*-maleimidehexane. Unlike forming a disulfide-bond between two free cysteines in the previous example, the *bis*-maleimidehexane crosslinker material should be stable to reducing agents such as β-mercaptoethanol. Figure 26 shows that approximately 5% of the treated material produced dimer with a molecular weight of 55,000 Daltons on a reducing SDS-PAGE gel (samples were treated with β-mercaptalethanol prior to being loaded on the gel). We further purified the *bis*-maleimidehexane treated 4-4-20/212 CPPC protein on PolyCAT A cation exchange column after the protein had been extensively dialyzed against buffer A. Figure 26 shows that we were able to enhance the fraction containing the dimer to approximately 15%.

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(54) Title: MULTIVALENT ANTIGEN-BINDING PROTEINS

(57) Abstract

Compositions of, genetic constructions coding for, and methods for producing multivalent antigen-binding proteins are described and claimed. The methods include purification of compositions containing both monomeric and multivalent forms of single polypeptide chain molecules, and production of multivalent proteins from purified monomers. Production of multivalent proteins may occur by a concentration-dependent association of monomeric proteins, or by rearrangement of regions involving dissociation followed by reassociation of different regions. Bivalent proteins, including homobivalent and heterobivalent proteins, are made in the present invention. Genetic sequences coding for bivalent single-chain antigen-binding proteins are disclosed. Uses include all those appropriate for monoclonal and polyclonal antibodies and fragments thereof, including use as a bispecific antigen-binding molecule.

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Multivalent Antigen-Binding Proteins

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Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Patent Application Serial Number 07/796,936, filed Nov. 25, 1991, which is a continuation-in-part of U.S. Patent Application Serial No. 07/512,910 filed April 25, 1990, which is a continuation-in-part of Serial No. 07/299,617, filed Jan. 1, 1989, issued as U.S. Patent No. 4,946,778 (Ladner *et al.*), which was a continuation-in-part of Serial No. 092,110, filed Sept. 2, 1987, and Serial No. 902,971, filed Sept. 2, 1986, now abandoned, the contents of all of which are fully incorporated herein by reference.

Background of the Invention

1. Field of the Invention

The present invention relates generally to the production of antigen-binding molecules. More specifically, the invention relates to multivalent forms of antigen-binding proteins. Compositions of, genetic constructions for, methods of use, and methods for producing these multivalent antigen-binding proteins are disclosed.

2. Description of the Background Art

Antibodies are proteins generated by the immune system to provide a specific molecule capable of complexing with an invading molecule, termed an antigen. Figure 14 shows the structure of a typical antibody molecule. Natural antibodies have two identical antigen-binding sites, both of which are specific to a particular antigen. The antibody molecule "recognizes" the antigen by complexing its antigen-binding sites with areas of the antigen termed epitopes. The epitopes fit into the conformational architecture of the antigen-binding sites of the antibody, enabling the antibody to bind to the antigen.

The antibody molecule is composed of two identical heavy and two identical light polypeptide chains, held together by interchain disulfide bonds (see Fig. 14). The remainder of this discussion will refer only to one light/heavy pair of chains, as each light/heavy pair is identical. Each individual light and heavy chain folds into regions of approximately 110 amino acids, assuming a conserved three-dimensional conformation. The light chain comprises one variable region (termed V_L) and one constant region (C_L), while the heavy chain comprises one variable region (V_H) and three constant regions (C_H1, C_H2 and C_H3). Pairs of regions associate to form discrete structures as shown in Figure 14. In particular, the light and heavy chain variable regions, V_L and V_H,associate to form an "F_V" area which contains the antigen-binding site.

The variable regions of both heavy and light chains show considerable variability in structure and amino acid composition from one antibody molecule to another, whereas the constant regions show little variability. The term "variable" as used in this specification refers to the diverse nature of the amino acid sequences of the antibody heavy and light chain variable regions. Each antibody recognizes and binds antigen through the binding site defined by the association of the heavy and light chain variable regions into an F_V area. The light-chain variable region V_L and the heavy-chain variable region V_H of a particular antibody molecule have specific amino acid sequences that

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allow the antigen-binding site to assume a conformation that binds to the antigen epitope recognized by that particular antibody.

Within the variable regions are found regions in which the amino acid sequence is extremely variable from one antibody to another. Three of these so-called "hypervariable" regions or "complementarity-determining regions" (CDR's) are found in each of the light and heavy chains. The three CDR's from a light chain and the three CDR's from a corresponding heavy chain form the antigen-binding site.

Cleavage of the naturally-occurring antibody molecule with the proteolytic enzyme papain generates fragments which retain their antigen-binding site. These fragments, commonly known as Fab's (for Fragment, antigen binding site) are composed of the C_L, V_L, C_H1 and V_H regions of the antibody. In the Fab the light chain and the fragment of the heavy chain are covalently linked by a disulfide linkage.

Recent advances in immunobiology, recombinant DNA technology, and computer science have allowed the creation of single polypeptide chain molecules that bind antigen. These single-chain antigen-binding molecules incorporate a linker polypeptide to bridge the individual variable regions, V_L and V_H, into a single polypeptide chain. A computer-assisted method for linker design is described more particularly in U.S. Patent No. 4,704,692, issued to Ladner *et al.* in November, 1987, and incorporated herein by reference. A description of the theory and production of single-chain antigen-binding proteins is found in U.S. Patent No. 4,946,778 (Ladner *et al.*), issued August 7, 1990, and incorporated herein by reference. The single-chain antigen-binding proteins produced under the process recited in U.S. Patent 4,946,778 have binding specificity and affinity substantially similar to that of the corresponding Fab fragment.

Bifunctional, or bispecific, antibodies have antigen binding sites of different specificities. Bispecific antibodies have been generated to deliver cells, cytotoxins, or drugs to specific sites. An important use has been to deliver host cytotoxic cells, such as natural killer or cytotoxic T cells, to specific cellular targets. (U.D. Staerz, O. Kanagawa, M.J. Bevan, *Nature*

314:628 (1985); S. Songilvilai, P.J. Lachmann, *Clin. Exp. Immunol.* 79: 315 (1990)). Another important use has been to deliver cytotoxic proteins to specific cellular targets. (V. Raso, T. Griffin, *Cancer Res. 41*:2073 (1981); S. Honda, Y. Ichimori, S. Iwasa, *Cytotechnology 4*:59 (1990)). Another important use has been to deliver anti-cancer non-protein drugs to specific cellular targets (J. Corvalan, W. Smith, V. Gore, *Intl. J. Cancer Suppl. 2*:22 (1988); M. Pimm *et al.*, *British J. of Cancer 61*:508 (1990)). Such bispecific antibodies have been prepared by chemical cross-linking (M. Brennan *et al.*, *Science 229*:81 (1985)), disulfide exchange, or the production of hybrid-hybridomas (quadromas). Quadromas are constructed by fusing hybridomas that secrete two different types of antibodies against two different antigens (Kurokawa, T. *et al.*, *Biotechnology 7*:1163 (1989)).

Summary of the Invention

This invention relates to the discovery that multivalent forms of single-chain antigen-binding proteins have significant utility beyond that of the monovalent single-chain antigen-binding proteins. A multivalent antigen-binding protein has more than one antigen-binding site. Enhanced binding activity, di- and multi-specific binding, and other novel uses of multivalent antigen-binding proteins have been demonstrated or are envisioned here. Accordingly, the invention is directed to multivalent forms of single-chain antigen-binding proteins, compositions of multivalent and single-chain antigen-binding proteins, methods of making and purifying multivalent forms of single-chain antigen-binding proteins, and uses for multivalent forms of single-chain antigen-binding proteins. The invention provides a multivalent antigen-binding protein comprising two or more single-chain protein molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain protein.

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Also provided is a composition comprising a multivalent antigen-binding protein substantially free of single-chain molecules.

Also provided is an aqueous composition comprising an excess of multivalent antigen-binding protein over single-chain molecules.

A method of producing a multivalent antigen-binding protein is provided, comprising the steps of producing a composition comprising multivalent antigen-binding protein and single-chain molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain molecule; separating the multivalent protein from the single-chain molecules; and recovering the multivalent protein.

Also provided is a method of producing multivalent antigen-binding protein, comprising the steps of producing a composition comprising single-chain molecules as previously defined; dissociating the single-chain molecules; reassociating the single-chain molecules; separating the resulting multivalent antigen-binding proteins from the single-chain molecules; and recovering the multivalent proteins.

Also provided is another method of producing a multivalent antigen-binding protein, comprising the step of chemically cross-linking at least two single-chain antigen-binding molecules.

Also provided is another method of producing a multivalent antigen-binding protein, comprising the steps of producing a composition comprising single-chain molecules as previously defined; concentrating said single-chain molecules; separating said multivalent protein from said single-chain molecules; and finally recovering said multivalent protein.

Also provided is another method of producing a multivalent antigen-binding protein comprising two or more single-chain molecules, each single-chain molecule as previously defined, said method comprising: providing a genetic sequence coding for said single-chain molecule; transforming a host

cell or cells with said sequence; expressing said sequence in said host or hosts; and recovering said multivalent protein.

Another aspect of the invention includes a method of detecting an antigen in or suspected of being in a sample, which comprises contacting said sample with the multivalent antigen-binding protein of claim 1 and detecting whether said multivalent antigen-binding protein has bound to said antigen.

Another aspect of the invention includes a method of imaging the internal structure of an animal, comprising administering to said animal an effective amount of a labeled form of the multivalent antigen-binding protein of claim 1 and measuring detectable radiation associated with said animal.

Another aspect of the invention includes a composition comprising an association of a multivalent antigen-binding protein with a therapeutically or diagnostically effective agent.

Another aspect of this invention is a single-chain protein comprising: a first polypeptide comprising the binding portion of the variable region of an antibody light chain; a second polypeptide comprising the binding portion of the variable region of an antibody light chain; a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

Another aspect of the present invention includes the genetic constructions encoding the combinations of regions V_L-V_L and V_H-V_H for single-chain molecules, and encoding multivalent antigen-binding proteins.

Another part of this invention is a multivalent single-chain antigen-binding protein comprising: a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein; a third polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and a peptide linker linking said second and third polypeptides (b) and (d) into said

multivalent protein. Also included are gentic constructions coding for this multivalent single-chain antigen-binding protein.

Also included are replicable cloning or expression vehicles including plasmids, hosts transformed with the aforementioned genetic sequences, and methods of producing multivalent proteins with the sequences, transformed hosts, and expression vehicles.

Methods of use are provided, such as a method of using the multivalent antigen-binding protein to diagnose a medical condition; a method of using the multivalent protein as a carrier to image the specific bodily organs of an animal; a therapeutic method of using the multivalent protein to treat a medical condition; and an immunotherapeutic method of conjugating a multivalent protein with a therapeutically or diagnostically effective agent. Also included are labelled multivalent proteins, improved immunoassays using them, and improved immunoaffinity purifications.

An advantage of using multivalent antigen-binding proteins instead of single-chain antigen-binding molecules or Fab fragments lies in the enhanced binding ability of the multivalent form. Enhanced binding occurs because the multivalent form has more binding sites per molecule. Another advantage of the present invention is the ability to use multivalent antigen-binding proteins as multi-specific binding molecules.

An advantage of using multivalent antigen-binding proteins instead of whole antibodies, is the enhanced clearing of the multivalent antigen-binding proteins from the serum due to their smaller size as compared to whole antibodies which may afford lower background in imaging applications. Multivalent antigen-binding proteins may penetrate solid tumors better than monoclonals, resulting in better tumor-fighting ability. Also, because they are smaller and lack the Fc component of intact antibodies, the multivalent antigen-binding proteins of the present invention may be less immunogenic than whole antibodies. The Fc component of whole antibodies also contains binding sites for liver, spleen and certain other cells and its absence should thus reduce accumulation in non-target tissues.

Another advantage of multivalent antigen-binding proteins is the ease with which they may be produced and engineered, as compared to the myeloma-fusing technique pioneered by Kohler and Milstein that is used to produce whole antibodies.

Brief Description of the Drawings.

The present invention as defined in the claims can be better understood with reference to the text and to the following drawings:

FIG. 1A is a schematic two-dimensional representation of two identical single-chain antigen-binding protein molecules, each comprising a variable light chain region (V_L), a variable heavy chain region (V_H), and a polypeptide linker joining the two regions. The single-chain antigen-binding protein molecules are shown binding antigen in their antigen-binding sites.

FIG. 1B depicts a hypothetical homodivalent antigen-binding protein formed by association of the polypeptide linkers of two monovalent single-chain antigen-binding proteins from Fig. 1A (the Association model). The divalent antigen-binding protein is formed by the concentration-driven association of two identical single-chain antigen-binding protein molecules.

FIG. 1C depicts the hypothetical divalent protein of FIG. 1B with bound antigen molecules occupying both antigen-binding sites.

FIG. 2A depicts the hypothetical homodivalent protein of Figure 1B.

FIG. 2B depicts three single-chain antigen-binding protein molecules associated in a hypothetical trimer.

FIG. 2C depicts a hypothetical tetramer of four single-chain antigen-binding protein molecules.

FIG. 3A depicts two separate and distinct monovalent single-chain antigen-binding proteins, Anti-A single-chain antigen-binding protein and Anti-B single-chain antigen-binding protein, with different antigen specificities, each individually binding either Antigen A or Antigen B.

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FIG. **3B** depicts a hypothetical bispecific heterodivalent antigen-binding protein formed from the single-chain antigen-binding proteins of Fig. 3A according to the Association model.

FIG. **3C** depicts the hypothetical heterodivalent antigen-binding protein of FIG. **3B** binding bispecifically, i.e., binding the two different antigens, A and B.

FIG. **4A** depicts two identical single-chain antigen-binding protein molecules, each having a variable light chain region (V_L), a variable heavy chain region (V_H), and a polypeptide linker joining the two regions. The single-chain antigen-binding protein molecules are shown binding identical antigen molecules in their antigen-binding sites.

FIG. **4B** depicts a hypothetical homodivalent protein formed by the rearrangement of the V_L and V_H regions shown in FIG. 4A (the Rearrangement model). Also shown is bound antigen.

FIG. **5A** depicts two single-chain protein molecules, the first having an anti-B V_L and an anti-A V_H, and the second having an anti-A V_L and an anti-B V_H. The figure shows the non-complementary nature of the V_L and V_H regions in each single-chain protein molecule.

FIG. **5B** shows a hypothetical bispecific heterodivalent antigen-binding protein formed by rearrangement of the two single-chain proteins of Figure 5A.

FIG. **5C** depicts the hypothetical heterodivalent antigen-binding protein of FIG. 5B with different antigens A and B occupying their respective antigen-binding sites.

FIG. **6A** is a schematic depiction of a hypothetical trivalent antigen-binding protein according to the Rearrangement model.

FIG. **6B** is a schematic depiction of a hypothetical tetravalent antigen-binding protein according to the Rearrangement model.

FIG. **7** is a chromatogram depicting the separation of CC49/212 antigen-binding protein monomer from dimer on a cation exchange high performance liquid chromatographic column. The column is a PolyCAT A

aspartic acid column (Poly WC, Columbia, MD). Monomer is shown as Peak 1, eluting at 27.32 min., and dimer is shown as Peak 2, eluting at 55.52 min.

FIG. **8** is a chromatogram of the purified monomer from Fig. 7. Monomer elutes at 21.94 min., preceded by dimer (20.135 min.) and trimer (18.640 min.). Gel filtration column, Protein-Pak 300SW (Waters Associates, Milford, MA).

FIG. **9** is a similar chromatogram of purified dimer (20.14 min.) from Fig. 7, run on the gel filtration HPLC column of Fig. 8.

FIG. **10A** is an amino acid (SEQ ID NO. 11) and nucleotide (SEQ ID NO. 10) sequence of the single-chain protein comprising the 4-4-20 V_L region connected through the 212 linker polypeptide to the CC49 V_H region.

FIG. **10B** is an amino acid (SEQ ID NO. 13) and nucleotide (SEQ ID NO. 12) sequence of the single-chain protein comprising the CC49 V_L region connected through the 212 linker polypeptide to the 4-4-20 V_H region.

FIG. **11** is a chromatogram depicting the separation of the monomer (27.83 min.) and dimer (50.47 min.) forms of the CC49/212 antigen-binding protein by cation exchange, on a PolyCAT A cation exchange column (Poly LC, Columbia, MD).

Fig. **12** shows the separation of monomer (17.65 min.), dimer (15.79 min.), trimer (14.19 min.), and higher oligomers (shoulder at about 13.09 min.) of the B6.2/212 antigen-binding protein. This separation depicts the results of a 24-hour treatment of a 1.0 mg/ml B6.2/212 single-chain antigen-binding protein sample. A TSK G2000SW gel filtration HPLC column was used, Toyo Soda, Tokyo, Japan.

Fig. **13** shows the results of a 24-hour treatment of a 4.0 mg/ml CC49/212 antigen-binding protein sample, generating monomer, dimer, and trimer at 16.91, 14.9, and 13.42 min., respectively. The same TSK gel filtration column was used as in Fig. 12.

Fig. **14** shows a schematic view of the four-chain structure of a human IgG molecule.

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Fig. **15A** is an amino acid (SEQ ID NO. 15) and nucleotide (SEQ ID NO. 14) sequence of the 4-4-20/212 single-chain antigen-binding protein with a single cysteine hinge.

Fig. **15B** is an amino acid (SEQ ID NO. 17) and nucleotide (SEQ. ID NO. 16) sequence of the 4-4-20/212 single-chain antigen-binding protein with the two-cysteine hinge.

Fig. **16** shows the amino acid (SEQ ID NO. 19) and nucleotide (SEQ ID NO. 18) sequence of a divalent CC49/212 single-chain antigen-binding protein.

Fig. **17** shows the expression of the divalent CC49/212 single-chain antigen-binding protein of Fig. 16 at 42°C, on an SDS-PAGE gel containing total *E. coli* protein. Lane 1 contains the molecular weight standards. Lane 2 is the uninduced *E. coli* production strain grown at 30°C. Lane 3 is divalent CC49/212 single-chain antigen-binding protein induced by growth at 42°C. The arrow shows the band of expressed divalent CC49/212 single-chain antigen-binding protein.

Fig. **18** is a graphical representation of four competition radioimmunoassays (RIA) in which unlabeled CC49 IgG (open circles) CC49/212 single-chain antigen-binding protein (closed circles) and CC49/212 divalent antigen-binding protein (closed squares) and anti-fluorescein 4-4-20/212 single-chain antigen-binding protein (open squares) competed against a CC49 IgG radiolabeled with ^{125}I for binding to the TAG-72 antigen on a human breast carcinoma extract.

Figure **19A** is an amino acid (SEQ ID NO. 21) and nucleotide (SEQ ID NO. 20) sequence of the single-chain polypeptide comprising the 4-4-20 V_L region connected through the 217 linker polypeptide to the CC49 V_H region.

Figure **19B** is an amino acid (SEQ ID NO. 23) and nucleotide (SEQ ID NO. 22) sequence of the single-chain polypeptide comprising the CC49 V_L region connected through the 217 linker polypeptide to the 4-4-20 V_H region.

Figure **20** is a chromatogram depicting the purification of CC49/4-4-20 heterodimer Fv on a cation exchange high performance liquid chromatographic column. The column is a PolyCAT A aspartic acid column (Poly LC,

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Columbia, MD). The heterodimer Fv is shown as peak 5, eluting at 30.10 min.

Figure **21** is a coomassie-blue stained 4-20% SDS-PAGE gel showing the proteins separated in Figure 20. Lane 1 contains the molecular weight standards. Lane 3 contains the starting material before separation. Lanes 4-8 contain fractions 2, 3, 5, 6 and 7 respectively. Lane 9 contains purified CC49/212.

Figure **22A** is a chromatogram used to determine the molecular size of fraction 2 from Figure **20**. A TSK G3000SW gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **22B** is a chromatogram used to determine the molecular size of fraction 5 from Figure **20**. A TSK G3000SW gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **22C** is a chromatogram used to determine the molecular size of fraction 6 from Figure **20**. A TSK G30005W gel filtration HPLC column was used (Toyo Soda, Tokyo, Japan).

Figure **23** shows a Scatchard analysis of the fluorescein binding affinity of the CC49 4-4-20 heterodimer Fv (fraction 5 in Figure **20**).

Figure **24** is a graphical representation of three competition enzyme-linked immunosorbent assays (ELISA) in which unlabeled CC49 4-4-20 Fv (closed squares) CC49/212 single-chain Fv (open squares) and MOPC-21 IgG (+) competed against a biotin-labeled CC49 IgG for binding to the TAG-72 antigen on a human breast carcinoma extract. MOPC-21 is a control antibody that does not bind to TAG-72 antigen.

Figure **25** shows a coomassie-blue stained non-reducing 4-20% SDS-PAGE gel. Lanes 1 and 9 contain the molecular weight standards. Lane 3 contains the 4-4-20/212 CPPC single-chain antigen-binding protein after purification. Lane 4, 5 and 6 contain the 4-4-20/212 CPPC single-chain antigen-binding protein after treatment with DTT and air oxidation. Lane 7 contains 4-4-20/212 single-chain antigen-binding protein.

Figure **26** shows a coomassie-blue stained reducing 4-20% SDS-PAGE gel (samples were treated with β-mercaptoethanol prior to being loaded on the

gel). Lanes 1 and 8 contain the molecular weight standards. Lane 3 contains the 4-4-20/212 CPPC single-chain antigen-binding protein after treatment with *bis*-maleimidehexane. Lane 5 contains peak 1 of *bis*-maleimidehexane treated 4-4-20/212 CPCC single-chain antigen-binding protein. Lane 6 contains peak 3 of *bis*-maleimidehexane treated 4-4-20/212 CPPC single-chain antigen-binding protein.

Detailed Description of the Preferred Embodiments

This invention relates to the discovery that multivalent forms of single-chain antigen-binding proteins have significant utility beyond that of the monovalent single-chain antigen-binding proteins. A multivalent antigen-binding protein has more than one antigen-binding site. For the purposes of this application, "valent" refers to the numerosity of antigen binding sites. Thus, a bivalent protein refers to a protein with two binding sites. Enhanced binding activity, bi- and multi-specific binding, and other novel uses of multivalent antigen-binding proteins have been demonstrated or are envisioned here. Accordingly, the invention is directed to multivalent forms of single-chain antigen-binding proteins, compositions of multivalent and single-chain antigen-binding proteins, methods of making and purifying multivalent forms of single-chain antigen-binding proteins, and new and improved uses for multivalent forms of single-chain antigen-binding proteins. The invention provides a multivalent antigen-binding protein comprising two or more single-chain protein molecules, each single-chain molecule comprising a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and a peptide linker linking the first and second polypeptides into a single-chain protein.

The term "multivalent" means any assemblage, covalently or non-covalently joined, of two or more single-chain proteins, the assemblage having more than one antigen-binding site. The single-chain proteins composing the

assemblage may have antigen-binding activity, or they may lack antigen-binding activity individually but be capable of assembly into active multivalent antigen-binding proteins. The term "multivalent" encompasses bivalent, trivalent, tetravalent, etc. It is envisioned that multivalent forms above bivalent may be useful for certain applications.

A preferred form of the multivalent antigen-binding protein comprises bivalent proteins, including heterobivalent and homobivalent forms. The term "bivalent" means an assemblage of single-chain proteins associated with each other to form two antigen-binding sites. The term "heterobivalent" indicates multivalent antigen-binding proteins that are bispecific molecules capable of binding to two different antigenic determinants. Therefore, heterobivalent proteins have two antigen-binding sites that have different binding specificities. The term "homobivalent" indicates that the two binding sites are for the same antigenic determinant.

The terms "single-chain molecule" or "single-chain protein" are used interchangeably here. They are structurally defined as comprising the binding portion of a first polypeptide from the variable region of an antibody, associated with the binding portion of a second polypeptide from the variable region of an antibody, the two polypeptides being joined by a peptide linker linking the first and second polypeptides into a single polypeptide chain. The single polypeptide chain thus comprises a pair of variable regions connected by a polypeptide linker. The regions may associate to form a functional antigen-binding site, as in the case wherein the regions comprise a light-chain and a heavy-chain variable region pair with appropriately paired complementarity determining regions (CDRs). In this case, the single-chain protein is referred to as a "single-chain antigen-binding protein" or "single-chain antigen-binding molecule."

Alternatively, the variable regions may have unnaturally paired CDRs or may both be derived from the same kind of antibody chain, either heavy or light, in which case the resulting single-chain molecule may not display a functional antigen-binding site. The single-chain antigen-binding protein

molecule is more fully described in U.S. Patent No. 4,946,778 (Ladner *et al.*), and incorporated herein by reference.

Without being bound by any particular theory, the inventors speculate on several models which can equally explain the phenomenon of multivalence. The inventors' models are presented herein for the purpose of illustration only, and are not to be construed as limitations upon the scope of the invention. The invention is useful and operable regardless of the precise mechanism of multivalence.

Figure 1 depicts the first hypothetical model for the creation of a multivalent protein, the "Association" model. Fig. 1A shows two monovalent single-chain antigen-binding proteins, each composed of a V_L, a V_H, and a linker polypeptide covalently bridging the two. Each monovalent single-chain antigen-binding protein is depicted having an identical antigen-binding site containing antigen. Figure 1B shows the simple association of the two single-chain antigen-binding proteins to create the bivalent form of the multivalent protein. It is hypothesized that simple hydrophobic forces between the monovalent proteins are responsible for their association in this manner. The origin of the multivalent proteins may be traceable to their concentration dependence. The monovalent units retain their original association between the V_H and V_L regions. Figure 1C shows the newly-formed homobivalent protein binding two identical antigen molecules simultaneously. Homobivalent antigen-binding proteins are necessarily monospecific for antigen.

Homovalent proteins are depicted in Figs. 2A through 2C formed according to the Association model. Fig. 1A depicts a homobivalent protein, Fig. 2B a trivalent protein, and Fig. 2C a tetravalent protein. Of course, the limitations of two-dimensional images of three-dimensional objects must be taken into account. Thus, the actual spatial arrangement of multivalent proteins can be expected to vary somewhat from these figures.

A heterobivalent antigen-binding protein has two different binding sites, the sites having different binding specificities. Figures 3A through C depict the Association model pathway to the creation of a heterobivalent protein. Figure 3A shows two monovalent single-chain antigen-binding proteins, Anti-

A single-chain antigen-binding protein and Anti-B single-chain antigen-binding protein, with antigen types A and B occupying the respective binding sites. Figure 3B depicts the heterobivalent protein formed by the simple association of the original monovalent proteins. Figure 3C shows the heterobivalent protein having bound antigens A and B into the antigen-binding sites. Figure 3C therefore shows the heterobivalent protein binding in a bispecific manner.

An alternative model for the formation of multivalent antigen-binding proteins is shown in Figures 4 through 6. This "Rearrangement" model hypothesizes the dissociation of the variable region interface by contact with dissociating agents such as guanidine hydrochloride, urea, or alcohols such as ethanol, either alone or in combination. Combinations and relevant concentration ranges of dissociating agents are recited in the discussion concerning dissociating agents, and in Example 2. Subsequent re-association of dissociated regions allows variable region recombination differing from the starting single-chain proteins, as depicted in Fig. 4B. The homobivalent antigen-binding protein of Figure 4B is formed from the parent single-chain antigen-binding proteins shown in Figure 4A, the recombined bivalent protein having V_L and V_H from the parent monovalent single-chain proteins. The homobivalent protein of Figure 4B is a fully functional monospecific bivalent protein, shown actively binding two antigen molecules.

Figures 5A-5C show the formation of heterobivalent antigen-binding proteins via the Rearrangement model. Figure 5A shows a pair of single-chain proteins, each having a V_L with complementarity determining regions (CDRs) that do not match those of the associated V_H. These single-chain proteins have reduced or no ability to bind antigen because of the mixed nature of their antigen-binding sites, and thus are made specifically to be assembled into multivalent proteins through this route. Figure 5B shows the heterobivalent antigen-binding protein formed whereby the V_H and V_L regions of the parent proteins are shared between the separate halves of the heterobivalent protein. Figure 5C shows the binding of two different antigen molecules to the resultant functional bispecific heterobivalent protein. The Rearrangement model also explains the generation of multivalent proteins of

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a higher order than bivalent, as it can be appreciated that more than a pair of single-chain proteins can be reassembled in this manner. These are depicted in Figures 6A and 6B.

One of the major utilities of the multivalent antigen-binding protein is in the heterobivalent form, in which one specificity is for one type of hapten or antigen, and the second specificity is for a second type of hapten or antigen. A multivalent molecule having two distinct binding specificities has many potential uses. For instance, one antigen binding site may be specific for a cell-surface epitope of a target cell, such as a tumor cell or other undesirable cell. The other antigen-binding site may be specific for a cell-surface epitope of an effector cell, such as the CD3 protein of a cytotoxic T-cell. In this way, the heterobivalent antigen-binding protein may guide a cytotoxic cell to a particular class of cells that are to be preferentially attacked.

Other uses of heterobivalent antigen-binding proteins are the specific targeting and destruction of blood clots by a bispecific molecule with specificity for tissue plasminogen activator (tPA) and fibrin; the specific targeting of pro-drug activating enzymes to tumor cells by a bispecific molecule with specificity for tumor cells and enzyme; and specific targeting of cytotoxic proteins to tumor cells by a bispecific molecule with specificity for tumor cells and a cytotoxic protein. This list is illustrative only, and any use for which a multivalent specificity is appropriate comes within the scope of this invention.

The invention also extends to uses for the multivalent antigen-binding proteins in purification and biosensors. Affinity purification is made possible by affixing the multivalent antigen-binding protein to a support, with the antigen-binding sites exposed to and in contact with the ligand molecule to be separated, and thus purified. Biosensors generate a detectable signal upon binding of a specific antigen to an antigen-binding molecule, with subsequent processing of the signal. Multivalent antigen-binding proteins, when used as the antigen-binding molecule in biosensors, may change conformation upon binding, thus generating a signal that may be detected.

Essentially all of the uses for which monoclonal or polyclonal antibodies, or fragments thereof, have been envisioned by the prior art, can be addressed by the multivalent proteins of the present invention. These uses include detectably-labelled forms of the multivalent protein. Types of labels are well-known to those of ordinary skill in the art. They include radiolabelling, chemiluminescent labeling, fluorochromic labelling, and chromophoric labeling. Other uses include imaging the internal structure of an animal (including a human) by administering an effective amount of a labelled form of the multivalent protein and measuring detectable radiation associated with the animal. They also include improved immunoassays, including sandwich immunoassay, competitive immunoassay, and other immunoassays wherein the labelled antibody can be replaced by the multivalent antigen-binding protein of this invention.

A first preferred method of producing multivalent antigen-binding proteins involves separating the multivalent proteins from a production composition that comprises both multivalent and single-chain proteins, as represented in Example 1. The method comprises producing a composition of multivalent and single-chain proteins, separating the multivalent proteins from the single-chain proteins, and recovering the multivalent proteins.

A second preferred method of producing multivalent antigen-binding proteins comprises the steps of producing single-chain protein molecules, dissociating said single-chain molecules, reassociating the single-chain molecules such that a significant fraction of the resulting composition includes multivalent forms of the single-chain antigen-binding proteins, separating multivalent antigen-binding proteins from single-chain molecules, and recovering the multivalent proteins. This process is illustrated with more detail in Example 2. For the purposes of this method, the term "producing a composition comprising single-chain molecules" may indicate the actual production of these molecules. The term may also include procuring them from whatever commercial or institutional source makes them available. Use of the term "producing single-chain proteins" means production of single-chain proteins by any process, but preferably according to the process set forth in

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U.S. Patent No. 4,946,778 (Ladner *et al.*). Briefly, that patent pertains to a single polypeptide chain antigen-binding molecule which has binding specificity and affinity substantially similar to the binding specificity and affinity of the aggregate light and heavy chain variable regions of an antibody, to genetic sequences coding therefore, and to recombinant DNA methods of producing such molecules, and uses for such molecules. The single-chain protein produced by the Ladner *et al.* methodology comprises two regions linked by a linker polypeptide. The two regions are termed the V_H and V_L regions, each region comprising one half of a functional antigen-binding site.

The term "dissociating said single-chain molecules" means to cause the physical separation of the two variable regions of the single-chain protein without causing denaturation of the variable regions.

"Dissociating agents" are defined herein to include all agents capable of dissociating the variable regions, as defined above. In the context of this invention, the term includes the well-known agents alcohol (including ethanol), guanidine hydrochloride (GuHCl), and urea. Others will be apparent to those of ordinary skill in the art, including detergents and similar agents capable of interrupting the interactions that maintain protein conformation. In the preferred embodiment, a combination of GuHCl and ethanol (EtOH) is used as the dissociating agent. A preferred range for ethanol and GuHCl is from 0 to 50% EtOH, vol/vol, 0 to 2.0 moles per liter (M) GuHCl. A more preferred range is from 10-30% EtOH and 0.5-1.0 M GuHCl, and a most preferred range is 20% EtOH, 0.5 M GuHCl. A preferred dissociation buffer contains 0.5 M guanidine hydrochloride, 20% ethanol, 0.05 M TRIS, and 0.01 M $CaCl_2$, pH 8.0.

Use of the term "re-associating said single-chain molecules" is meant to describe the reassociation of the variable regions by contacting them with a buffer solution that allows reassociation. Such a buffer is preferably used in the present invention and is characterized as being composed of 0.04 M MOPS, 0.10 M calcium acetate, pH 7.5. Other buffers allowing the reassociation of the V_L and V_H regions are well within the expertise of one of ordinary skill in the art.

The separation of the multivalent protein from the single-chain molecules occurs by use of standard techniques known in the art, particularly including cation exchange or gel filtration chromatography.

Cation exchange chromatography is the general liquid chromatographic technique of ion-exchange chromatography utilizing anion columns well-known to those of ordinary skill in the art. In this invention, the cations exchanged are the single-chain and multivalent protein molecules. Since multivalent proteins will have some multiple of the net charge of the single-chain molecule, the multivalent proteins are retained more strongly and are thus separated from the single-chain molecules. The preferred cationic exchanger of the present invention is a polyaspartic acid column, as shown in Figure 7. Figure 7 depicts the separation of single-chain protein (Peak 1, 27.32 min.) from bivalent protein (Peak 2, 55.54 min.) Those of ordinary skill in the art will realize that the invention is not limited to any particular type of chromatography column, so long as it is capable of separating the two forms of protein molecules.

Gel filtration chromatography is the use of a gel-like material to separate proteins on the basis of their molecular weight. A "gel" is a matrix of water and a polymer, such as agarose or polymerized acrylamide. The present invention encompasses the use of gel filtration HPLC (high performance liquid chromatography), as will be appreciated by one of ordinary skill in the art. Figure 8 is a chromatogram depicting the use of a Waters Associates' Protein-Pak 300 SW gel filtration column to separate monovalent single-chain protein from multivalent protein, including the monomer (21.940 min.), bivalent protein (20.135 min.), and trivalent protein (18.640 min.).

Recovering the multivalent antigen-binding proteins is accomplished by standard collection procedures well known in the chemical and biochemical arts. In the context of the present invention recovering the multivalent protein preferably comprises collection of eluate fractions containing the peak of interest from either the cation exchange column, or the gel filtration HPLC column. Manual and automated fraction collection are well-known to one of

ordinary skill in the art. Subsequent processing may involve lyophilization of the eluate to produce a stable solid, or further purification.

A third preferred method of producing multivalent antigen-binding proteins is to start with purified single-chain proteins at a lower concentration, and then increase the concentration until some significant fraction of multivalent proteins is formed. The multivalent proteins are then separated and recovered. The concentrations conducive to formation of multivalent proteins in this manner are from about 0.5 milligram per milliliter (mg/ml) to the concentration at which precipitates begin to form.

The use of the term "substantially free" when used to describe a composition of multivalent and single-chain antigen-binding protein molecules means the lack of a significant peak corresponding to the single-chain molecule, when the composition is analyzed by cation exchange chromatography, as disclosed in Example 1 or by gel filtration chromatography as disclosed in Example 2.

By use of the term "aqueous composition" is meant any composition of single-chain molecules and multivalent proteins including a portion of water. In the same context, the phrase "an excess of multivalent antigen-binding protein over single-chain molecules" indicates that the composition comprises more than 50% of multivalent antigen-binding protein.

The use of the term "cross-linking" refers to chemical means by which one can produce multivalent antigen-binding proteins from monovalent single-chain protein molecules. For example, the incorporation of a cross-linkable sulfhydryl chemical group as a cysteine residue in the single-chain proteins allows cross-linking by mild reduction of the sulfhydryl group. Both monospecific and multispecific multivalent proteins can be produced from single-chain proteins by cross-linking the free cysteine groups from two or more single-chain proteins, causing a covalent chemical linkage to form between the individual proteins. Free cysteines have been engineered into the C-terminal portion of the 4-4-20/212 single-chain antigen-binding protein, as discussed in Example 5 and Example 8. These free cysteines may then be cross-linked to form multivalent antigen-binding proteins.

The invention also comprises single-chain proteins, comprising: (a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain; (b) a second polypeptide comprising the binding portion of the variable region of an antibody light chain; and (c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein. A similar single-chain protein comprising the heavy chain variable regions is also a part of this invention. Genetic sequences encoding these molecules are also included in the scope of this invention. Since these proteins are comprised of two similar variable regions, they do not necessarily have any antigen-binding capability.

The invention also includes a DNA sequence encoding a bispecific bivalent antigen-binding protein. Example 4 and Example 7 discusses in detail the sequences that appear in Figs. 10A and 10B that allow one of ordinary skill to construct a heterobivalent antigen-binding molecule. Figure 10A is an amino acid and nucleotide sequence listing of the single-chain protein comprising the 4-4-20 V_L region connected through the 212 linker polypeptide to the CC49 V_H region. Figure 10B is a similar listing of the single-chain protein comprising the CC49 V_L region connected through the 212 linker polypeptide to the 4-4-20 V_H region. Subjecting a composition including these single-chain molecules to dissociating and subsequent re-associating conditions results in the production of a bivalent protein with two different binding specificities.

Synthesis of DNA sequences is well know in the art, and possible through at least two routes. First, it is well-known that DNA sequences may be synthesized through the use of automated DNA synthesizers *de novo*, once the primary sequence information is known. Alternatively, it is possible to obtain a DNA sequence coding for a multivalent single-chain antigen-binding protein by removing the stop codons from the end of a gene encoding a single-chain antigen-binding protein, and then inserting a linker and a gene encoding a second single-chain antigen-binding protein. Example 6 demonstrates the construction of a DNA sequence coding for a bivalent single-chain antigen-binding protein. Other methods of genetically constructing multivalent single-

chain antigen-binding proteins come within the spirit and scope of the present invention.

Having now generally described this invention the same will better be understood by reference to certain specific examples which are included for purposes of illustration and are not intended to limit it unless otherwise specified.

Example 1

Production of Multivalent Antigen-Binding Proteins During Purification

In the production of multivalent antigen-binding proteins, the same recombinant *E. coli* production system that was used for prior single-chain antigen-binding protein production was used. See Bird, *et al.*, *Science* **242**:423 (1988). This production system produced between 2 and 20% of the total *E. coli* protein as antigen-binding protein. For protein recovery, the frozen cell paste from three 10-liter fermentations (600-900 g) was thawed overnight at 4°C and gently resuspended at 4°C in 50 mM Tris-Hcl, 1.0 mM EDTA, 100 mM KCl, 0.1 mM PMSF, pH 8.0 (lysis buffer), using 10 liters of lysis buffer for every kilogram of wet cell paste. When thoroughly resuspended, the chilled mixture was passed three times through a Manton-Gaulin cell homogenizer to totally lyse the cells. Because the cell homogenizer raised the temperature of the cell lysate to 25 ±5°C, the cell lysate was cooled to 5±2°C with a Lauda/Brinkman chilling coil after each pass. Complete lysis was verified by visual inspection under a microscope.

The cell lysate was centrifuged at 24,300g for 30 min. at 6°C using a Sorvall RC-5B centrifuge. The pellet containing the insoluble antigen-binding protein was retained, and the supernatant was discarded. The pellet was washed by gently scraping it from the centrifuge bottles and resuspending it in 5 liters of lysis buffer/kg of wet cell paste. The resulting 3.0- to 4.5-liter suspension was again centrifuged at 24,300g for 30 min at 6°C, and the

supernatant was discarded. This washing of the pellet removes soluble *E. coli* proteins and can be repeated as many as five times. At any time during this washing procedure the material can be stored as a frozen pellet at -20°C. A substantial time saving in the washing steps can be accomplished by utilizing a Pellicon tangential flow apparatus equipped with 0.22-μm microporous filters, in place of centrifugation.

The washed pellet was solubilized at 4°C in freshly prepared 6 M guanidine hydrochloride, 50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, pH 8.0 (dissociating buffer), using 9 ml/g of pellet. If necessary, a few quick pulses from a Heat Systems Ultrasonics tissue homogenizer can be used to complete the solubilization. The resulting suspension was centrifuged at 24,300g for 45 min at 6°C and the pellet was discarded. The optical density of the supernatant was determined at 280 nm and if the OD_{280} was above 30, additional dissociating buffer was added to obtain an OD_{280} of approximately 25.

The supernatant was slowly diluted into cold (4-7°C) refolding buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 50 mM KCl, pH 8.0) until a 1:10 dilution was reached (final volume 10-20 liters). Re-folding occurs over approximately eighteen hours under these conditions. The best results are obtained when the GuHCl extract is slowly added to the refolding buffer over a 2-h period, with gentle mixing. The solution was left undisturbed for at least a 20-h period, and 95% ethanol was added to this solution such that the final ethanol concentration was approximately 20%. This solution was left undisturbed until the flocculated material settled to the bottom, usually not less than sixty minutes. The solution was filtered through a 0.2 um Millipore Millipak 200. This filtration step may be optionally preceded by a centrifugation step. The filtrate was concentrated to 1 to 2 liters using an Amicon spiral cartridge with a 10,000 MWCO cartridge, again at 4°C.

The concentrated crude antigen-binding protein sample was dialyzed against Buffer A (60 mM MOPS, 0.5 mM Ca acetate, pH 6.0-6.4) until the conductivity was lowered to that of Buffer A. The sample was then loaded on a 21.5 x 250-mm polyaspartic acid PolyCAT A column, manufactured by Poly

LC of Columbia, Maryland. If more than 60 mg of protein is loaded on this column, the resolution begins to deteriorate; thus, the concentrated crude sample often must be divided into several PolyCAT A runs. Most antigen-binding proteins have an extinction coefficient of about 2.0 ml mg^{-1} cm^{-1} at 280 nm and this can be used to determine protein concentration. The antigen-binding protein sample was eluted from the PolyCAT A column with a 50-min linear gradient from Buffer A to Buffer B (see Table 1). Most of the single-chain proteins elute between 20 and 26 minutes when this gradient is used. This corresponds to an eluting solvent composition of approximately 70% Buffer A and 30% Buffer B. Most of the bivalent antigen-binding proteins elute later than 45 minutes, which correspond to over 90% Buffer B.

Figure 7 is a chromatogram depicting the separation of single-chain protein from bivalent CC49/212 protein, using the cation-exchange method just described. Peak 1, 27.32 minutes, represents the monomeric single-chain fraction. Peak 2, 55.52 minutes, represents the bivalent protein fraction.

Figure 8 is a chromatogram of the purified monomeric single-chain antigen-binding protein CC49/212 (Fraction 7 from Fig. 7) run on a Waters Protein-Pak 300SW gel filtration column. Monomer, with minor contaminates of dimer and trimer, is shown. Figure 9 is a chromatogram of the purified bivalent antigen-binding protein CC49/212 (Fraction 15 from Fig. 7) run on the same Waters Protein-Pak 300SW gel filtration column as used in Fig. 8.

cation exchange and gel filtration chromatography, can be used to separate the single-chain protein monomer from the multivalent antigen-binding proteins. In the first method, monomeric and multivalent antigen-binding proteins were separated by using cation exchange HPLC chromography, using a polyaspartate column (PolyCAT A). This was a similar procedure to that used in the final purification of the antigen-binding proteins as described in Example 1. The load buffer was 0.06 M MOPS, 0.001 M Calcium Acetate pH 6.4. In the second method, the monomeric and multivalent antigen-binding proteins were separated by gel filtration HPLC chromatography using as a load buffer 0.04 M MOPS, 0.10 M Calcium Acetate pH 7.5. Gel filtration chromatography separates proteins based on their molecular size.

Once the antigen-binding protein sample was loaded on the cation exchange HPLC column, a linear gradient was run between the load buffer (0.04 to 0.06 M MOPS, 0.000 to 0.001 M calcium acetate, 0 to 10% glycerol pH 6.0-6.4) and a second buffer (0.04 to 0.06 M MOPS, 0.01 to 0.02 M calcium acetate, 0 to 10% glycerol pH 7.5). It was important to have extensively dialyze the antigen-binding protein sample before loading it on the column. Normally, the conductivity of the sample is monitored against the dialysis buffer. Dialysis is continued until the conductivity drops below 600 μS. Figure 11 shows the separation of the monomeric (27.83 min.) and bivalent (50.47 min.) forms of the CC49/212 antigen-binding protein by cation exchange. The chromatographic conditions for this separation were as follows: PolyCAT A column, 200 x 4.6mm, operated at 0.62 ml/min.; load buffer and second buffer as in Example 1; gradient program from 100 percent load buffer A to 0 percent load buffer A over 48 mins; sample was CC49/212, 1.66 mg/ml; injection volume 0.2 ml. Fractions were collected from the two peaks from a similar chromatogram and identified as monomeric and bivalent proteins using gel filtration HPLC chromatography as described below.

Gel filtration HPLC chromatography (TSK G2000SW column from Toyo Soda, Tokyo, Japan) was used to identify and separate monomeric single-chain and multivalent antigen-binding proteins. This procedure has been described by Fukano, *et al., J. Chromatography* 166:47 (1978).

Multimerization (creation of multivalent protein from monomeric single-chain protein) was by treatment with 0.5 M GuHCl and 20% EtOH for the times indicated in Table 2A followed by dialysis into the chromatography buffer. Figure 12 shows the separation of monomeric (17.65 min.), bivalent (15.79 min.), trivalent (14.19 min.), and higher oligomers (shoulder at about 13.09 min.) of the B6.2/212 antigen-binding protein. The B6.2/212 single-chain antigen-binding protein is described in Colcher, D., *et al.*, *J. Nat. Cancer Inst. 82*:1191-1197 (1990)). This separation depicts the results of a 24-hour multimerization treatment of a 1.0 mg/ml B6.2/212 antigen-binding protein sample. The HPLC buffer used was 0.04 M MOPS, 0.10 M calcium acetate, 0.04% sodium azide, pH 7.5.

Figure 13 shows the results of a 24-hour treatment of a 4.0 mg/ml CC49/212 antigen-binding protein sample, generating monomeric, bivalent and trivalent proteins at 16.91, 14.9, and 13.42 min., respectively. The HPLC buffer was 40 mM MOPS, 100 mM calcium acetate, pH 7.35. Multimerization treatment was for the times indicated in Table 2.

The results of Example 2A are shown in Table 2A. Table 2A shows the percentage of bivalent and other multivalent forms before and after treatment with 20% ethanol and 0.5M GuHCl. Unless otherwise indicated, percentages were determined using a automatic data integration software package.

Table 2A
Summary of the generation of bivalent and higher multivalent forms of B6.2/212 and CC49/212 proteins using guanidine hydrochloride and ethanol

protein	Time (hours)	Concentration (mg/ml)	% monomer	dimer	trimer	multimers
CC49/212	0	0.25	86.7	11.6	1.7	0.0
	0	1.0[2]	84.0	10.6	5.5	0.0
	0	4.0	70.0	17.1	12.9[1]	0.0
	2	0.25[2]	62.9	33.2	4.2	0.0
	2	1.0	24.2	70.6	5.1	0.0
	2	4.0	9.3	81.3	9.5	0.0
	26	0.25	16.0	77.6	6.4	0.0
	26	1.0	9.2	82.8	7.9	0.0
	26	4.0	3.7	78.2	18.1	0.0
B6.2/212	0	0.25	100.0	0.0	0.0	0.0
	0	1.0	100.0	0.0	0.0	0.0
	0	4.0	100.0	0.0	0.0	0.0
	2	0.25[2]	98.1	1.9	0.0	0.0
	2	1.0	100.0	0.0	0.0	0.0
	2	4.0	90.0	5.5	1.0	0.0
	24	0.25	45.6	37.5	10.2	6.7
	24	1.0	50.8	21.4	12.3	15.0
	24	4.0	5.9	37.2	25.7	29.9

[1] Based on cut out peaks that were weighted.
[2] Average of two experiments.

B. Process Using Urea and Ethanol

Multivalent antigen-binding proteins were produced from purified single-chain proteins in the following way. First the purified single-chain protein at a concentration of 0.25-1 mg/ml was dialyzed against 2M urea, 20% ethanol (EtOH), and 50mM Tris buffer pH 8.0, for the times indicated in Table 2B. This combination of dissociating agents is thought to disrupt the V_L/V_H interface, allowing the V_H of a first single-chain molecule to come into contact with a V_L from a second single-chain molecule. Other dissociating agents such as isopropanol or methanol should be substitutable for EtOH.

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Following the initial dialysis, the protein was dialyzed against the load buffer for the final HPLC purification step.

Gel filtration HPLC chromatography (TSK G2000SW column from Toyo Soda, Tokyo, Japan) was used to identify and separate monomeric single-chain and multivalent antigen-binding proteins. This procedure has been described by Fukano, *et al., J. Chromatography* **166**:47 (1978).

The results of Example 2B are shown in Table 2B. Table 2B shows the percentage of bivalent and other multivalent forms before and after treatment with 20% ethanol and urea. Percentages were determined using an automatic data integration software package.

Table 2B

Summary of the generation of bivalent and higher multivalent forms of B6.2/212 and CC49/212 proteins using urea and ethanol

protein	Time (hours)	Concentration (mg/ml)	% monomer	% dimer	% trimer	% multimers
B6.2	0	0.25	44.1	37.6	15.9	2.4
	0	1.0	37.7	33.7	19.4	9.4
	3	0.25	22.2	66.5	11.3	0.0
	3	1.0	13.7	69.9	16.4	0.0

Example 3

Determination of Binding Constants

Three anti-fluorescein single-chain antigen-binding proteins have been constructed based on the anti-fluorescein monoclonal antibody 4-4-20. The three 4-4-20 single-chain antigen-binding proteins differ in the polypeptide linker connecting the V_H and V_L regions of the protein. The three linkers used were 202', 212 and 216 (see Table 3). Bivalent and higher forms of the 4-4-20 antigen-binding protein were produced by concentrating the purified monomeric single-chain antigen-binding protein in the cation exchange load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) to 5 mg/ml. The

bivalent and monomeric forms of the 4-4-20 antigen-binding proteins were separated by cation exchange HPLC (polyaspartate column) using a 50 min. linear gradient between the load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) and a second buffer (0.06 M MOPS, 0.02 M calcium acetate pH 7.5). Two 0.02 ml samples were separated, and fractions of the bivalent and monomeric protein peaks were collected on each run. The amount of protein contained in each fraction was determined from the absorbance at 278 nm from the first separation. Before collecting the fractions from the second separation run, each fraction tube had a sufficient quantity of 1.03×10^{5} M fluorescein added to it, such that after the fractions were collected a 1-to-1 molar ratio of protein-to-fluorescein existed. Addition of fluorescein stabilized the bivalent form of the 4-4-20 antigen-binding proteins. These samples were kept at 2°C (on ice).

The fluorescein dissociation rates were determined for each of these samples following the procedures described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe,* E.W. Voss, Ed., CRC Press, Boca Raton, FL (1984). A sample was first diluted with 20 mM HEPES buffer pH 8.0 to 5.0×10^{-8} M 4-4-20 antigen-binding protein. 560 μl of the 5.0×10^{-8} M 4-4-20 antigen-binding protein sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at 2°C and the fluorescence was read. 140 μl of 1.02×10^{-5} M fluoresceinamine was added to the cuvette, and the fluorescence was read every 1 minute for up to 25 minutes (see Table 4).

The binding constants (K_a) for the 4-4-20 single-chain antigen-binding protein monomers diluted in 20 mM HEPES buffer pH 8.0 in the absence of fluorescein were also determined (see Table 4).

The three polypeptide linkers in these experiments differ in length. The 202', 212 and 216 linkers are 12, 14 and 18 residues long, respectively. These experiments show that there are two effects of linker length on the 4-4-20 antigen-binding proteins: first, the shorter the linker length the higher the fraction of bivalent protein formed; second, the fluorescein dissociation rates of the monomeric single-chain antigen-binding proteins are effected more by the linker length than are the dissociation rates of the bivalent antigen-binding

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proteins. With the shorter linkers 202' and 212, the bivalent antigen-binding proteins have slower dissociation rates than the monomers. Thus, the linkers providing optimum production and binding affinities for monomeric and bivalent antigen-binding proteins may be different. Longer linkers may be more suitable for monomeric single-chain antigen-binding proteins, and shorter linkers may be more suitable for multivalent antigen-binding proteins.

Table 3

Linker Designs

V_L	Linker	V_H	Linker Name	Reference
-KLEIE	GKSSGSGSESKS[1]	TQKLD-	202'	Bird *et al.*
-KLEIK	GSTSGSGKSSEGKG[2]	EVKLD-	212	Bedzyk *et al.*
-KLEIK	GSTSGSGKSSEGSGSTKG[3]	EVKLD-	216	This application
-KLVLK	GSTSGKPSEGKG[4]	EVKLD-	217	This application

(1) SEQ ID NO. 1
(2) SEQ ID NO. 2
(3) SEQ ID NO. 3
(4) SEQ ID NO. 4

Table 4

Effects of Linkers on the SCA Protein Monomers and Dimers

	Linker		
	202'	212	216
Monomer			
Fraction	0.47	0.66	0.90
Ka	$0.5 \times 10^9\ M^{-1}$	$1.0 \times 10^9\ M^{-1}$	$1.3 \times 10^9\ M^{-1}$
Dissociation rate	$8.2 \times 10^{-3}\ s^{-1}$	$4.9 \times 10^{-3}\ s^{-1}$	$3.3 \times 10^{-3}\ s^{-1}$
Dimer			
Fraction	0.53	0.34	0.10
Dissociation rate	$4.6 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$
Monomer/Dimer			
Dissociation rate ratio	1.8	1.4	0.9

Example 4

bivalent and monomeric forms of the 4-4-20 antigen-binding proteins were separated by cation exchange HPLC (polyaspartate column) using a 50 min. linear gradient between the load buffer (0.06 M MOPS, 0.001 M calcium acetate pH 6.4) and a second buffer (0.06 M MOPS, 0.02 M calcium acetate pH 7.5). Two 0.02 ml samples were separated, and fractions of the bivalent and monomeric protein peaks were collected on each run. The amount of protein contained in each fraction was determined from the absorbance at 278 nm from the first separation. Before collecting the fractions from the second separation run, each fraction tube had a sufficient quantity of 1.03×10^{5} M fluorescein added to it, such that after the fractions were collected a 1-to-1 molar ratio of protein-to-fluorescein existed. Addition of fluorescein stabilized the bivalent form of the 4-4-20 antigen-binding proteins. These samples were kept at 2°C (on ice).

The fluorescein dissociation rates were determined for each of these samples following the procedures described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe,* E.W. Voss, Ed., CRC Press, Boca Raton, FL (1984). A sample was first diluted with 20 mM HEPES buffer pH 8.0 to 5.0×10^{-8} M 4-4-20 antigen-binding protein. 560 μl of the 5.0×10^{-8} M 4-4-20 antigen-binding protein sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at 2°C and the fluorescence was read. 140 μl of 1.02×10^{-5} M fluoresceinamine was added to the cuvette, and the fluorescence was read every 1 minute for up to 25 minutes (see Table 4).

The binding constants (K_a) for the 4-4-20 single-chain antigen-binding protein monomers diluted in 20 mM HEPES buffer pH 8.0 in the absence of fluorescein were also determined (see Table 4).

The three polypeptide linkers in these experiments differ in length. The 202', 212 and 216 linkers are 12, 14 and 18 residues long, respectively. These experiments show that there are two effects of linker length on the 4-4-20 antigen-binding proteins: first, the shorter the linker length the higher the fraction of bivalent protein formed; second, the fluorescein dissociation rates of the monomeric single-chain antigen-binding proteins are effected more by the linker length than are the dissociation rates of the bivalent antigen-binding

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proteins. With the shorter linkers 202' and 212, the bivalent antigen-binding proteins have slower dissociation rates than the monomers. Thus, the linkers providing optimum production and binding affinities for monomeric and bivalent antigen-binding proteins may be different. Longer linkers may be more suitable for monomeric single-chain antigen-binding proteins, and shorter linkers may be more suitable for multivalent antigen-binding proteins.

Table 3

Linker Designs

V_L	Linker	V_H	Linker Name	Reference
-KLEIE	GKSSGSGSESKS[1]	TQKLD-	202'	Bird *et al.*
-KLEIK	GSTSGSGKSSEGKG[2]	EVKLD-	212	Bedzyk *et al.*
-KLEIK	GSTSGSGKSSEGSGSTKG[3]	EVKLD-	216	This application
-KLVLK	GSTSGKPSEGKG[4]	EVKLD-	217	This application

(1) SEQ ID NO. 1
(2) SEQ ID NO. 2
(3) SEQ ID NO. 3
(4) SEQ ID NO. 4

Table 4

Effects of Linkers on the SCA Protein Monomers and Dimers

	Linker		
	202'	212	216
Monomer			
Fraction	0.47	0.66	0.90
Ka	$0.5 \times 10^9\ M^{-1}$	$1.0 \times 10^9\ M^{-1}$	$1.3 \times 10^9\ M^{-1}$
Dissociation rate	$8.2 \times 10^{-3}\ s^{-1}$	$4.9 \times 10^{-3}\ s^{-1}$	$3.3 \times 10^{-3}\ s^{-1}$
Dimer			
Fraction	0.53	0.34	0.10
Dissociation rate	$4.6 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$	$3.5 \times 10^{-3}\ s^{-1}$
Monomer/Dimer			
Dissociation rate ratio	1.8	1.4	0.9

Example 4

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Genetic Construction of a Mixed-Fragment Bivalent Antigen-Binding Protein

The genetic constructions for one particular heterobivalent antigen-binding protein according to the Rearrangement model are shown in Figures 10A and 10B. Figure 10A is an amino acid and nucleotide sequence listing of the 4-4-20 V_L/212/CC49 V_H construct, coding for a single-chain protein with a 4-4-20 V_L, linked via a 212 polypeptide linker to a CC49 V_H. Figure 10B is a similar listing showing the CC49 V_L/212/4-4-20 V_H construct, coding for a single-chain protein with a CC49 V_L, linked via a 212 linker to a 4-4-20 V_H. These single-chain proteins may recombine according to the Rearrangement model to generate a heterobivalent protein comprising a CC49 antigen-binding site linked to a 4-4-20 antigen-binding site, as shown in Figure 5B.

"4-4-20 V_L" means the variable region of the light chain of the 4-4-20 mouse monoclonal antibody (Bird, R.E. *et al., Science* **242:**423 (1988)). The number "212" refers to a specific 14-residue polypeptide linker that links the 4-4-20 V_L and the CC49 V_H. See *Bedzyk, W.D.* et al., *J. Biol. Chem. 265:18615-18620 (1990).* "CC49 V_H" is the variable region of the heavy chain of the CC49 antibody, which binds to the TAG-72 antigen. The CC49 antibody was developed at The National Institutes of Health by Schlom, et al. *Generation and Characterization of B72.3 Second Generation Monoclonal Antibodies Reactive With The Tumor-associated Glycoprotein 72 Antigen,* Cancer Research 48:4588-4596 (1988).

Insertion of the sequences shown in FIGS. 10A and 10B, by standard recombinant DNA methodology, into a suitable plasmid vector will enable one of ordinary skill in the art to transform a suitable host for subsequent expression of the single-chain proteins. See Maniatis et al., *Molecular Cloning, A Laboratory Manual*, p. 104, Cold Spring Harbor Laboratory (1982), for general recombinant techniques for accomplishing the aforesaid goals; see also U.S. Patent 4,946,778 (Ladner *et al.*) for a complete

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description of methods of producing single-chain protein molecules by recombinant DNA technology.

To produce multivalent antigen-binding proteins from the two single-chain proteins, 4-4-20V_L-212/CC49V_H and CC49V_L/212/4-4-20V_H, the two single-chain proteins are dialyzed into 0.5 M GuHCl/20% EtOH being combined in a single solution either before or after dialysis. The multivalent proteins are then produced and separated as described in Example 2.

Example 5

Preparation of Multivalent Antigen-Binding Proteins by Chemical Cross-Linking

Free cysteines were engineered into the C-terminal of the 4-4-20/212 single-chain antigen-binding protein, in order to chemically crosslink the protein. The design was based on the hinge region found in antibodies between the C_H1 and C_H2 regions. In order to try to reduce antigenicity in humans, the hinge sequence of the most common IgG class, IgG1, was chosen. The 4-4-20 Fab structure was examined and it was determined that the C-terminal sequence GluH216-ProH217-ArgH218, was part of the C_H1 region and that the hinge between C_H1 and C_H2 starts with ArgH218 or GlyH219 in the mouse 4-4-20 IgG2A antibody. Figure 14 shows the structure of a human IgG. The hinge region is indicated generally. Thus the hinge from human IgG1 would start with LysH218 or SerH219. (See Table 5).

The C-terminal residue in most of the single-chain antigen-binding proteins described to date is the amino acid serine. In the design for the hinge region, the C-terminal serine in the 4-4-20/212 single-chain antigen-binding protein was made the first serine of the hinge and the second residue of the hinge was changed from a cysteine to a serine. This hinge cysteine normally forms a disulfide bridge to the C-terminal cysteine in the light chain.

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TABLE 5

	218
IgG2A mouse[1]	E P R G P T I K P C P P C L C -
IgG1 human[2]	A E P K S C D K T H T C P P C -
SCA[3]	- - V T V S
SCA* Hinge design 1[4]	- - V T V S S D K T H T C
SCA* Hinge design 2[5]	- - V T V S S D K T H T C P P C

* - single-chain antigen-binding protein

(1) SEQ ID NO. 5
(2) SEQ ID NO. 6
(3) SEQ ID NO. 7
(4) SEQ ID NO. 8
(5) SEQ ID NO. 9

There are possible advantages to having two C-terminal cysteines, for they might form an intramolecular disulfide bond, making the protein recovery easier by protecting the sulfurs from oxidation. The hinge regions were added by introduction of a BstE II restriction site in the 3'-terminus of the gene encoding the 4-4-20/212 single-chain antigen-binding protein (see Figures 15A-15B).

The monomeric single-chain antigen-binding protein containing the C-terminal cysteine can be purified using the normal methods of purifying a single-chain antigen-binding proteins, with minor modifications to protect the free sulfhydryls. The cross-linking could be accomplished in one of two ways. First, the purified single-chain antigen-binding protein could be treated with a mild reducing agent, such as dithiothreitol, then allowed to air oxidize to form a disulfide-bond between the individual single-chain antigen-binding proteins. This type of chemistry has been successful in producing heterodimers from whole antibodies (Nisonoff *et al.*, Quantitative Estimation of the Hybridization of Rabbit Antibodies, *Nature 4826*:355-359 (1962); Brennan *et al.*, Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments, *Science 229*:81-83 (1985)). Second, chemical crosslinking agents such as *bis*maleimidehexane could be used to cross-link two single-chain antigen-binding proteins by their C-terminal cysteines. See Partis *et al.*, *J. Prot. Chem.* 2:263-277 (1983).

Example 6

Genetic Construction of Bivalent Antigen-Binding Proteins

Bivalent antigen-binding proteins can be constructed genetically and subsequently expressed in *E. coli* or other known expression systems. This can be accomplished by genetically removing the stop codons at the end of a gene encoding a monomeric single-chain antigen-binding protein and inserting a linker and a gene encoding a second single-chain antigen-binding protein. We have constructed a gene for a bivalent CC49/212 antigen-binding protein in this manner (see Figure 16). The CC49/212 gene in the starting expression plasmid is in an Aat II to Bam H1 restriction fragment (see Bird *et al.*, Single-Chain Antigen-Binding Proteins, *Science 242:*423-426 (1988); and Whitlow *et al.*, Single-Chain F_V Proteins and Their Fusion Proteins, *Methods* 2:97-105 (1991)). The two stop codons and the Bam H1 site at the C-terminal end of the CC49/212 antigen-binding protein gene were replaced by a single residue linker (Ser) and an Aat II restriction site. The resulting plasmid was cut with Aat II and the purified Aat II to Aat II restriction fragment was ligated into Aat II cut CC49/212 single-chain antigen-binding protein expression plasmid. The resulting bivalent CC49/212 single-chain antigen-binding protein expression plasmid was transfected into an *E. coli* expression host that contained the gene for the cI857 temperature-sensitive repressor. Expression of single-chain antigen-binding protein in this system is induced by raising the temperature from 30°C to 42°C. Fig. 17 shows the expression of the divalent CC49/212 single-chain antigen-binding protein of Fig. 16 at 42°C, on an SDS-PAGE gel containing total *E. coli* protein. Lane 1 contains the molecular weight standards. Lane 2 is the uninduced *E. coli* production strain grown at 30°C. Lane 3 is divalent CC49/212 single-chain antigen-binding protein induced by growth at 42°C. The arrow shows the band of expressed divalent CC49/212 single-chain antigen-binding protein.

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Example 7

Construction, Purification, and Testing of 4-4-20/CC49 Heterodimer F_V With 217 Linkers.

The goals of this experiment were to produce, purify and analyze for activity a new heterodimer Fv that would bind to both fluorescein and the pan-carcinoma antigen TAG-72. The design consisted of two polypeptide chains, which associated to form the active heterodimer Fv. Each polypeptide chain can be described as a mixed single-chain Fv (mixed sFv). The first mixed sFv (GX 8952) comprised a 4-4-20 variable light chain (V_L) and a CC-49 variable heavy chain (V_H) connected by a 217 polypeptide linker (Figure 19A). The second mixed sFv (GX 8953) comprised a CC-49 V_L and a 4-4-20 V_H connected by a 217 polypeptide linker (Figure 19B). The sequence of the 217 polypeptide linker is shown in Table 3. Construction of analogous CC49/4-4-20 heterodimers connected by a 212 polypeptide linker as described in Example 4.

Results

A. Purification

One 10-liter fermentation of each mixed sFv was grown on casein digest-glucose-salts medium at 32°C to an optical density at 600 nm of 15 to 20. The mixed sFv expression was induced by raising the temperature of the fermentation to 42°C for one hour. 277gm (wet cell weight) of *E. coli* strain GX 8952 and 233gm (wet cell weight) of *E. coli* strain GX 8953 were harvested in a centrifuge at 7000g for 10 minutes. The cell pellets were kept and the supernate discarded. The cell pellets were frozen at -20°0C for storage.

2.55 liters of "lysis/wash buffer" (50mM Tris/ 200mM NaCl/ 1 mM EDTA, pH 8.0) was added to both of the mixed sFv's cell pellets, which were previously thawed and combined to give 510gm of total wet cell weight. After complete suspension of the cells they were then passed through a Gaulin homogenizer at 9000psi and 4°C. After this first pass the temperature increased to 23°C. The temperature was immediately brought down to 0°C using dry ice and methanol. The cell suspension was passed through the Gaulin homogenizer a second time and centrifuged at 8000 rpm with a Dupont GS-3 rotor for 60 minutes. The supernatant was discarded after centrifugation and the pellets resuspended in 2.5 liters of "lysis/wash buffer" at 4°C. This suspension was centrifuged for 45 minutes at 8000 rpm with the Dupont GS-3 rotor. The supernatant was again discarded and the pellet weighed. The pellet weight was 136.1 gm.

1300ml of 6M Guanidine Hydrochloride/50mM Tris/50mM KCl/10mM $CaCl_2$ pH 8.0 at 4°C was added to the washed pellet. An overhead mixer was used to speed solubilization. After one hour of mixing, the heterodimer GuHCl extract was centrifuged for 45 minutes at 8000 rpm and the pellet was discarded. The 1425ml of heterodimer Fv 6M GuHCl extract was slowly added (16 ml/min) to 14.1 liters of "Refold Buffer" (50mM Tris/50mM KCl/10mM $CaCl_2$, pH 8.0) under constant mixing at 4°C to give an approximate dilution of 1:10. Refolding took place overnight at 4°C.

After 17 hours of refolding the anti-fluorescein activity was checked by a 40% quenching assay, and the amount of active protein calculated. 150mg total active heterodimer Fv was found by the 40% quench assay, assuming a 54,000 molecular weight.

4 liters of prechilled (4°C) 190 proof ethanol was added to the 15 liters of refolded heterodimer with mixing for 3 hours. The mixture sat overnight at 4°C. A flocculent precipitate had settled to the bottom after this overnight treatment. The nearly clear solution was filtered through a Millipak-200 (0.22μ) filter so as to not disturb the precipitate. A 40% quench assay showed that 10% of the anti-fluorescein activity was recovered in the filtrate.

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The filtered sample of heterodimer was dialyzed, using a Pellicon system containing 10,000 dalton MWCO membranes, with "dialysis buffer" 40mM MOPS/0.5mM Calcium Acetate (CaAc), pH 6.4 at 4°C. 20 liters of dialysis buffer was required before the conductivity of the retentate was equal to that of the dialysis buffer (~500μS). After dialysis the heterodimer sample was filtered through a Millipak-20 filter, 0.22μ. After this step a 40% quench assay showed there was 8.8 mg of active protein.

The crude heterodimer sample was loaded on a Poly CAT A cation exchange column at 20ml/min. The column was previously equilibrated with 60mM MOPS, 1 mM CaAc pH 6.4, at 4°C, (Buffer A). After loading, the column was washed with 150ml of "Buffer A" at 15ml/min. A 50min linear gradient was performed at 15ml/min using "Buffer A" and "Buffer B" (60mM MOPS, 20mM CaAc pH 7.5 at 4°C). The gradient conditions are presented in Table 6. "Buffer C" comprises 60mM MOPS, 100mM $CaCl_2$, pH 7.5.

Table 6

Time	%A	%B	%C	Flow
0:00	100.0	0.0	0.0	15ml/min
50:00	0.0	100.0	0.0	15ml/min
52:00	0.0	100.0	0.0	15ml/min
54:00	0.0	0.0	100.0	15ml/min
58:00	0.0	0.0	100.0	15ml/min
60:00	100.0	0.0	0.0	15ml/min

Approximately 50ml fractions were collected and analyzed for activity, purity, and molecular weight by size-exclusion chromatography. The fractions were not collected by peaks, so contamination between peaks is likely. Fractions 3 through 7 were pooled (total volume - 218ml), concentrated to 50ml and dialyzed against 4 liters of 60mM MOPS, 0.5mM CaAc pH 6.4 at 4°C overnight. The dialyzed pool was filtered through a 0.22μ filter and

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checked for absorbance at 280nm. The filtrate was loaded onto the PolyCAT A column, equilibrated with 60mM MOPS, 1 mM CaAc pH 6.4 at 4°C, at a flow rate of 10ml/min. Buffer B was changed to 60mM MOPS, 10mM CaAc pH 7.5 at 4°C. The gradient was run as in Table 6. The fractions were collected by peak and analyzed for activity, purity, and molecular weight. The chromatogram is shown in Figure 20. Fraction identification and analysis is presented in Table 7.

Table 7

Fraction Analysis of the Heterodimer Fv protein

Fraction No.	A_{280} reading	Total Volume (ml)	HPLC-SE Elution Time (min)
2	0.161	36	20.525
3	0.067	40	
4	0.033	40	
5	0.178	45	19.133
6	0.234	50	19.163
7	0.069	50	
8	0.055	40	

Fractions 2 to 7 and the starting material were analyzed by SDS gel electrophoresis, 4-20%. A picture and description of the gel is presented in Figure 21.

B. HPLC Size Exclusion Results

Fractions 2, 5, and 6 correspond to the three main peaks in Figure 20 and therefore were chosen to be analyzed by HPLC size exclusion. Fraction 2 corresponds to the peak that runs at 21.775 minutes in the preparative purification (Figure 20), and runs on the HPLC sizing column at 20.525 minutes, which is in the monomeric position (Figure 22A). Fractions 5 and 6 (30.1 and 33.455 minutes, respectively, in Figure 20) run on the HPLC sizing column (Figures 22B and 22C) at 19.133 and 19.163 minutes,

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respectively (see Table 7). Therefore, both of these peaks could be considered dimers. 40% Quenching assays were performed on all fractions of this purification. Only fraction 5 gave significant activity. 2.4 mg of active CC49 4-4-20 heterodimer Fv was recovered in fraction 5, based on the Scatchard analysis described below.

C. N-terminal sequencing of the fractions

The active heterodimer Fv fraction should contain both polypeptide chains. N-terminal sequence analysis showed that fractions 5 and 6 displayed N-terminal sequences consistent with the prescence of both CC49 and 4-4-20 polypeptides and fraction 2 displayed a single sequence corresponding to the CC49/212/4-4-20 polypeptide only. We believe that fraction 6 was contaminated by fraction 5 (see Figure 20), since only fraction 5 had significant activity.

D. Anti-fluorescein activity by Scatchard analysis

The fluorescein association constants (Ka) were determined for fractions 5 and 6 using the fluorescence quenching assay described by Herron, J.N., in *Fluorescence Hapten: An Immunological Probe*, E.W. Voss, ed., CRC Press, Boca Raton, FL (1984). Each sample was diluted to approximately 5.0 x 10^{-8} M with 20 mM HEPES buffer pH 8.0. 590 μl of the 5.0 x 10^{-8} M sample was added to a cuvette in a fluorescence spectrophotometer equilibrated at room temperature. In a second cuvette 590 μl of 20 mM HEPES buffer pH 8.0 was added. To each cuvette was added 10 μl of 3.0 x 10^{-7} M fluorescein in 20 mM HEPES buffer pH 8.0, and the fluorescence recorded. This is repeated until 140 μl of fluorescein had been added. The resulting Scatchard analysis for fraction 5 shows a binding constant of 1.16 x 10^{9} M^{-1} for fraction #5 (see Figure 23). This is very close to the 4-4-20/212 sFv constant of 1.1 x 10^{9} M^{-1} (see Pantoliano *et al.*, *Biochemistry 30*:10117-10125 (1991)). The R intercept on the Scatchard analysis represents the fraction of active material. For fraction 5, 61% of the

material was active. The graph of the Scatchard analysis on fraction 6 shows a binding constant of 3.3 x 10^8 M^{-1} and 14% active. The activity that is present in fraction 6 is most likely contaminants from fraction 5.

E. Anti-TAG-72 activity by competition ELISA

The CC49 monoclonal antibody was developed by Dr. Jeffrey Schlom's group, Laboratory of Tumor Immunology and Biology, National Cancer Institute. It binds specifically to the pan-carcinoma tumor antigen TAG-72. See Muraro, R., *et al.*, *Cancer Research 48*:4588-4596 (1988).

To determine the binding properties of the bivalent CC49/4-4-20 Fv (fraction 5) and the CC49/212 sFv, a competition enzyme-linked immunosorbent assay (ELISA) was set up in which a CC49 IgG labeled with biotin was competed against unlabeled CC49/4-4-20 Fv and the CC49/212 sFv for binding to TAG-72 on a human breast carcinoma extract (see Figure 24). The amount of biotin-labeled CC49 IgG was determined using a preformed complex with avidin and biotin coupled to horse radish peroxidase and O-phenylenediamine dihydrochloride (OPD). The reaction was stopped with 4N H_2SO_4 (sulfuric acid), after 10 min. and the optical density read at 490nm. This competition ELISA showed that the bivalent CC49/4-4-20 Fv binds to the TAG-72 antigen. The CC49/4-4-20 Fv needed a two hundred-fold higher protein concentration to displace the IgG than the single-chain Fv.

Example 8

Cross-Linking Antigen-Binding Dimers

We have chemically crosslinked dimers of 4-4-20/212 antigen-binding protein with the two cysteine C-terminal extension (4-4-20/212 CPPC single-chain antigen-binding protein) in two ways. In Example 5 we describe the design and genetic construction of the 4-4-20/212 CPPC single-chain antigen-binding protein (hinge design 2 in Table 5). Figure 15B shows the nucleic

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acid and protein sequences of this protein. After purifying the 4-4-20/212 CPPC single-chain antigen-binding protein, using the methods described in Whitlow and Filpula, *Meth. Enzymol. 2*:97 (1991), dimers were formed by two methods. First, the free cysteines were mildly reduced with dithiothreitol (DTT) and then the disulfide-bonds between the two molecules were allowed to form by air oxidation. Second, the chemical crosslinker *bis*-maleimidehexane was used to produce dimers by crosslinking the free cysteines from two 4-4-20/212 CPPC single-chain antigen-binding proteins.

A 0.1 mg/ml solution of the 4-4-20/212 CPPC single-chain antigen-binding protein was mildly reduced using 1 mM DTT, 50 mM HEPES, 50mM NaCl, 1 mM EDTA buffer pH 8.0 at 4°C. The samples were dialyzed against 50mM HEPES, 50 mM NaCl, 1 mM EDTA buffer pH 8.0 at 4°C overnight, to allow the oxidation of free sulfhydrals to intermolecular disulfide-bonds. Figure 25 shows a non-reducing SDS-PAGE gel after the air oxidation; it shows that approximately 10% of the 4-4-20/212 CPPC protein formed dimers with molecular weights around 55,000 Daltons.

A 0.1 mg/ml solution of the 4-4-20/212 CPPC single-chain antigen-binding protein was treated with 2 mM *bis*-maleimidehexane. Unlike forming a disulfide-bond between two free cysteines in the previous example, the *bis*-maleimidehexane crosslinker material should be stable to reducing agents such as β-mercaptoethanol. Figure 26 shows that approximately 5% of the treated material produced dimer with a molecular weight of 55,000 Daltons on a reducing SDS-PAGE gel (samples were treated with β-mercaptalethanol prior to being loaded on the gel). We further purified the *bis*-maleimidehexane treated 4-4-20/212 CPPC protein on PolyCAT A cation exchange column after the protein had been extensively dialyzed against buffer A. Figure 26 shows that we were able to enhance the fraction containing the dimer to approximately 15%.

Conclusions

We have produced a heterodimer Fv from two complementary mixed sFv's which has been shown to have the size of a dimer of the sFv's. The N-terminal analysis has shown that the active heterodimer Fv contains two polypeptide chains. The heterodimer Fv has been shown to be active for both fluorescein and TAG-72 binding.

All publications cited herein are incorporated fully into this disclosure by reference.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention and the following claims. As examples, the steps of the preferred embodiment constitute only one form of carrying out the process in which the invention may be embodied.

SEQUENCE LISTING

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Lys Ser Ser Gly Ser Gly Ser Glu Ser Lys Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Ser Gly Ser Thr
1 5 10 15

Lys Gly

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Thr Ser Gly Lys Pro Ser Glu Gly Lys Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Pro Arg Gly Pro Thr Ile Lys Pro Cys Pro Pro Cys Leu Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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1 5 10 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: both

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 731 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..729

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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 1               5                  10                  15

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT      96
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

AAT GGA AAC ACC TAT TTA CGT TGG TAC CTG CAG AAG CCA GGC CAG TCT     144
Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA     192
Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC     240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT     288
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA     336
Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

GGT TCT ACC TCT GGT TCT GGT AAA TCC TCT GAA GGC AAA GGT CAG GTT     384
Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Gln Val
        115                 120                 125
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CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT GGG GCT TCA GTG                 432
Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser Val
    130                 135                 140

AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA ATT                 480
Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile
145                 150                 155                 160

CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT                 528
His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr
                165                 170                 175

TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG GGC                 576
Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly
            180                 185                 190

AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT GCC TAC GTG CAG                 624
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        195                 200                 205

CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC TGT ACA AGA                 672
Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr Arg
    210                 215                 220

TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC                 720
Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser
225                 230                 235                 240

TAA TAG GAT CC                                                                 731
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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 243 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
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        35                  40                  45

Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Gln Val
        115                 120                 125

Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser Val
    130                 135                 140

Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile
145                 150                 155                 160

His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr
                165                 170                 175
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Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly
180 185 190

Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val Gln
195 200 205

Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr Arg
210 215 220

Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser
225 230 235 240

* * Asp

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 744 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..744

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC 48
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly
1 5 10 15

GAG AAG GTT ACT TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT 96
Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
20 25 30

GGT AAT CAA AAG AAC TAC TTG GCC TGG TAC CAG CAG AAA CCA GGG CAG 144
Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45

TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG GAA TCT GGG GTC 192
Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
50 55 60

CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC 240
Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65 70 75 80

ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG 288
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
85 90 95

TAT TAT AGC TAT CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG 336
Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
100 105 110

AAA GGC TCT ACT TCC GGT AGC GGC AAA TCT TCT GAA GGT AAA GGT GAA 384
Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu
115 120 125

GTT AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT GGG AGG CCC 432
Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro
130 135 140

ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG 480
Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp
145 150 155 160

ATG AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA 528
Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala
165 170 175

CAA ATT AGA AAC AAA CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT 576
Gln Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser
180 185 190

ID: <WO___9311161A1_I_>

```
GTG AAA GGC AGA TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT AGT GTC        624
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val
        195   -             200                 205

TAC CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT ATC TAT TAC        672
Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr
    210             215                 220

TGT ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCA        720
Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser
225                 230                 235                 240

GTC ACC GTC TCC TAA TAA GGA TCC                                       744
Val Thr Val Ser  *   *  Gly Ser
                245
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 248 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly
  1               5                  10                  15

Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
    50                  55                  60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65                  70                  75                  80

Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                85                  90                  95

Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
            100                 105                 110

Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu
        115                 120                 125

Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro
    130                 135                 140

Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp
145                 150                 155                 160

Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala
                165                 170                 175

Gln Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser
            180                 185                 190

Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val
        195                 200                 205

Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr
    210                 215                 220

Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser
225                 230                 235                 240

Val Thr Val Ser  *   *  Gly Ser
                245
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 761 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..756

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT          48
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT          96
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

AAT GGA AAC ACC TAT TTA CGT TGG TAC CTG CAG AAG CCA GGC CAG TCT         144
Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA         192
Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC         240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT         288
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA         336
Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

GGT TCT ACC TCT GGT TCT GGT AAA TCT TCT GAA GGT AAA GGT GAA GTT         384
Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu Val
        115                 120                 125

AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT GGG AGG CCC ATG         432
Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met
    130                 135                 140

AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG         480
Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
145                 150                 155                 160

AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA         528
Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln
                165                 170                 175

ATT AGA AAC AAA CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG         576
Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val
            180                 185                 190

AAA GGC AGA TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT AGT GTC TAC         624
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr
        195                 200                 205

CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT ATC TAT TAC TGT         672
Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys
    210                 215                 220

ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCG GTC         720
Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
225                 230                 235                 240
```

```
ACC GTC TCC AGT GAT AAG ACC CAT ACA TGC TAA TAGGATCC                    761
Thr Val Ser Ser Asp Lys Thr His Thr Cys  *
                245                 250
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 251 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
  1               5                  10                  15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
             20                  25                  30

Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
         35                  40                  45

Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
     50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
 65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                 85                  90                  95

Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu Val
        115                 120                 125

Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met
    130                 135                 140

Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
145                 150                 155                 160

Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln
                165                 170                 175

Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val
            180                 185                 190

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr
        195                 200                 205

Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys
    210                 215                 220

Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
225                 230                 235                 240

Thr Val Ser Ser Asp Lys Thr His Thr Cys  *
                245                 250
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 770 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A)-NAME/KEY: CDS
(B) LOCATION: 1..765

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT          48
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT          96
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

AAT GGA AAC ACC TAT TTA CGT TGG TAC CTG CAG AAG CCA GGC CAG TCT         144
Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA         192
Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC         240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT         288
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA         336
Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

GGT TCT ACC TCT GGT TCT GGT AAA TCT TCT GAA GGT AAA GGT GAA GTT         384
Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu Val
        115                 120                 125

AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT GGG AGG CCC ATG         432
Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met
    130                 135                 140

AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG         480
Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
145                 150                 155                 160

AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA         528
Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln
                165                 170                 175

ATT AGA AAC AAA CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG         576
Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val
            180                 185                 190

AAA GGC AGA TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT AGT GTC TAC         624
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr
        195                 200                 205

CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT ATC TAT TAC TGT         672
Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys
    210                 215                 220

ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCG GTC         720
Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
225                 230                 235                 240

ACC GTC TCC AGT GAT AAG ACC CAT ACA TGC CCT CCA TGC TAA TAGGATCC         770
Thr Val Ser Ser Asp Lys Thr His Thr Cys Pro Pro Cys *
                245                 250                 255
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 254 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Glu Val
        115                 120                 125

Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met
    130                 135                 140

Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
145                 150                 155                 160

Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln
                165                 170                 175

Ile Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val
            180                 185                 190

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr
        195                 200                 205

Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys
    210                 215                 220

Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
225                 230                 235                 240

Thr Val Ser Ser Asp Lys Thr His Thr Cys Pro Pro Cys  *
                245                 250
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1460 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1398

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC          48
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly
 1               5                  10                  15

GAG AAG GTT ACT TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT          96
Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

GGT AAT CAA AAG AAC TAC TTG GCC TGG TAC CAG CAG AAA CCA GGG CAG         144
Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG GAA TCT GGG GTC         192
Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
    50                  55                  60

CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC         240
Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65                  70                  75                  80

ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG         288
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                85                  90                  95

TAT TAT AGC TAT CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG         336
Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
            100                 105                 110

AAA GGC TCT ACT TCC GGT AGC GGC AAA TCC TCT GAA GGC AAA GGT CAG         384
Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Gln
        115                 120                 125

GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT GGG GCT TCA         432
Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser
    130                 135                 140

GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA         480
Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala
145                 150                 155                 160

ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA         528
Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly
                165                 170                 175

TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG         576
Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
            180                 185                 190

GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT GCC TAC GTG         624
Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val
        195                 200                 205

CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC TGT ACA         672
Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr
    210                 215                 220


AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC         720
Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
225                 230                 235                 240

TCC TCA GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA         768
Ser Ser Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser
                245                 250                 255

GTT GGC GAG AAG GTT ACT TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA         816
Val Gly Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu
            260                 265                 270

TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC TGG TAC CAG CAG AAA CCA         864
Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
        275                 280                 285

GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG GAA TCT         912
Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser
    290                 295                 300
```

```
GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT        960
Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr
305             310                 315                 320

CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT       1008
Leu Ser Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys
            325                 330                 335

CAG CAG TAT TAT AGC TAT CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT       1056
Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu
        340                 345                 350

GTG CTG AAA GGC TCT ACT TCC GGT AGC GGC AAA TCC TCT GAA GGC AAA       1104
Val Leu Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys
    355                 360                 365

GGT CAG GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT GGG       1152
Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly
370                 375                 380

GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC       1200
Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp
385             390                 395                 400

CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG       1248
His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp
            405                 410                 415

ATT GGA TAT TTT TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG       1296
Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg
        420                 425                 430

TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT GCC       1344
Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala
    435                 440                 445

TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC       1392
Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
450                 455                 460

TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC       1440
Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val
465             470                 475                 480

ACC GTC TCC TAA TAG GAT CC                                            1460
Thr Val Ser  *   *  Asp
                485
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 486 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly
 1               5                  10                  15

Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
    50                  55                  60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65                  70                  75                  80
```

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Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Ser Cys Gln Gln
85 90 95

Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
100 105 110

Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys Gly Gln
115 120 125

Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser
130 135 140

Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala
145 150 155 160

Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly
165 170 175

Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
180 185 190

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val
195 200 205

Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr
210 215 220

Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
225 230 235 240

Ser Ser Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser
245 250 255

Val Gly Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu
260 265 270

Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
275 280 285

Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser
290 295 300

Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr
305 310 315 320

Leu Ser Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys
325 330 335

Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu
340 345 350

Val Leu Lys Gly Ser Thr Ser Gly Ser Gly Lys Ser Ser Glu Gly Lys
355 360 365

Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly
370 375 380

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp
385 390 395 400

His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp
405 410 415

Ile Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg
420 425 430

Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala
435 440 445

Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
450 455 460

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val
465 470 475 480

Thr Val Ser * * Asp
485

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 725 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..723

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT          48
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly
 1               5                  10                  15

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT          96
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

AAT GGA AAC ACC TAT TTA CGT TGG TAC CTG CAG AAG CCA GGC CAG TCT         144
Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT TCT GGG GTC CCA         192
Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC         240
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT         288
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

ACA CAT GTT CCG TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA         336
Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

GGT TCT ACC TCT GGT AAA CCA TCT GAA GGC AAA GGT CAG GTT CAG CTG         384
Gly Ser Thr Ser Gly Lys Pro Ser Glu Gly Lys Gly Gln Val Gln Leu
        115                 120                 125

CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT GGG GCT TCA GTG AAG ATT         432
Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile
    130                 135                 140

TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC TGG         480
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp
145                 150                 155                 160

GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT TTT TCT         528
Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe Ser
                165                 170                 175

CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG GGC AAG GCC         576
Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala
            180                 185                 190

ACA CTG ACT GCA GAC AAA TCC TCC AGC ACT GCC TAC GTG CAG CTC AAC         624
Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val Gln Leu Asn
        195                 200                 205

AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC TGT ACA AGA TCC CTG         672
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr Arg Ser Leu
    210                 215                 220
```

)OCID: <WO 9311161A1 | >

```
AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TAA TAG      720
Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser  *   *
225                 230                 235                 240

                                                                    725
GAT CC
Asp
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(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 241 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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 1               5                  10                  15

Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser
            20                  25                  30

Asn Gly Asn Thr Tyr Leu Arg Trp Tyr Leu Gln Lys Pro Gly Gln Ser
        35                  40                  45

Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
65                  70                  75                  80

Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser
                85                  90                  95

Thr His Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110

Gly Ser Thr Ser Gly Lys Pro Ser Glu Gly Lys Gly Gln Val Gln Leu
        115                 120                 125

Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile
    130                 135                 140

Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp
145                 150                 155                 160

Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe Ser
                165                 170                 175

Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala
            180                 185                 190

Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val Gln Leu Asn
        195                 200                 205

Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Thr Arg Ser Leu
    210                 215                 220

Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser  *   *
225                 230                 235                 240

Asp
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(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 738 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..738

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

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GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC          48
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly
 1               5                  10                  15

GAG AAG GTT ACT TGG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT          96
Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

GGT AAT CAA AAG AAC TAC TTG GCC TGG TAC CAG CAG AAA CCA GGG CAG         144
Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG GAA TCT GGG GTC         192
Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
    50                  55                  60

CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC         240
Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65                  70                  75                  80

ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG         288
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                85                  90                  95

TAT TAT AGC TAT CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG         336
Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
            100                 105                 110

AAA GGC TCT ACT TCC GGT AAA CCA TCT GAA GGT AAA GGT GAA GTT AAA         384
Lys Gly Ser Thr Ser Gly Lys Pro Ser Glu Gly Lys Gly Glu Val Lys
        115                 120                 125

CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT GGG AGG CCC ATG AAA         432
Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met Lys
    130                 135                 140

CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG AAC         480
Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met Asn
145                 150                 155                 160

TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA ATT         528
Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile
                165                 170                 175

AGA AAC AAA CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG AAA         576
Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys
            180                 185                 190

GGC AGA TTC ACC ATC TCA AGA GAT GAT TCC AAA AGT AGT GTC TAC CTG         624
Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr Leu
        195                 200                 205

CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT ATC TAT TAC TGT ACG         672
Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys Thr
    210                 215                 220

GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC         720
Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr
225                 230                 235                 240

GTC TCC TAA TAA GGA TCC                                                738
Val Ser  *   *  Gly Ser
                245
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(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 246 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

)OCID: <WO 9311161A1 | >

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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Glu Lys Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
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Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
35 40 45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val
50 55 60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
65 70 75 80

Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
85 90 95

Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu
100 105 110

Lys Gly Ser Thr Ser Gly Lys Pro Ser Glu Gly Lys Gly Glu Val Lys
115 120 125

Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly Arg Pro Met Lys
130 135 140

Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met Asn
145 150 155 160

Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile
165 170 175

Arg Asn Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys
180 185 190

Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Ser Ser Val Tyr Leu
195 200 205

Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile Tyr Tyr Cys Thr
210 215 220

Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr
225 230 235 240

Val Ser * * Gly Ser
245

What Is Claimed Is:

1. A multivalent antigen-binding protein comprising two or more single-chain molecules, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule.

2. The multivalent protein of claim 1 wherein said first polypeptide comprises the binding portion of the variable region of an antibody light chain, and said second polypeptide comprises the binding portion of the variable region of an antibody heavy chain.

3. The multivalent protein of claim 1 wherein said first polypeptide comprises the binding portion of the variable region of an antibody light chain, and said second polypeptide comprises the binding portion of the variable region of an antibody light chain.

4. The multivalent protein of claim 1 wherein said first polypeptide comprises the binding portion of the variable region of an antibody heavy chain, and said second polypeptide comprises the binding portion of the variable region of an antibody heavy chain.

5. The multivalent protein of claims 1, 2, 3, or 4 comprising a bivalent antigen-binding protein.

6. The multivalent protein of claim 5 comprising a heterobivalent antigen-binding protein.

7. The multivalent protein of claim 5 comprising a homobivalent antigen-binding protein.

8. A composition comprising a multivalent antigen-binding protein substantially free of single-chain molecules, wherein said multivalent protein comprises two or more single-chain molecules, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule.

9. The composition of claim 8 wherein said first polypeptide comprises the binding portion of the variable region of an antibody light chain, and said second polypeptide comprises the binding portion of the variable region of an antibody heavy chain.

10. The composition of claim 8 wherein said first polypeptide comprises the binding portion of the variable region of an antibody light chain, and said second polypeptide comprises the binding portion of the variable region of an antibody light chain.

11. The composition of claim 8 wherein said first polypeptide comprises the binding portion of the variable region of an antibody heavy chain, and said second polypeptide comprises the binding portion of the variable region of an antibody heavy chain.

12. The composition of claims 8, 9, 10, or 11, comprising a bivalent antigen-binding protein substantially free of single-chain molecules.

13. The composition of claim 12 wherein said bivalent protein is heterobivalent.

14. The composition of claim 12 wherein said bivalent protein is homobivalent.

15. An aqueous composition comprising an excess of multivalent antigen-binding protein over single-chain molecules, said multivalent protein comprising two or more single-chain molecules, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

16. The aqueous composition of claim 15 wherein at least one of said single-chain molecules comprises:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

17. The aqueous composition of claim 15 wherein at least one of said single-chain molecules comprises:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

18. The composition of claim 15 wherein at least one of said single-chain molecules comprises:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

19. A method of producing a multivalent antigen-binding protein, comprising the steps of:

(a) producing a composition comprising multivalent antigen-binding protein and single-chain molecules, each single-chain molecule comprising:

(i) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(ii) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(iii) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule;

(b) separating said multivalent protein from said single-chain molecules; and

(c) recovering said multivalent protein.

20. The method of claim 19 wherein separating said multivalent protein from said single-chain molecules comprises utilizing cation exchange chromatography.

21. The method of claim 19 wherein separating said multivalent protein from said single-chain molecules comprises utilizing gel filtration chromatography.

22. A method of producing a multivalent antigen-binding protein comprising the steps of:

(a) producing a composition comprising single-chain molecules, each single-chain molecule comprising:

(i) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(ii) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(iii) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule;

(b) dissociating said single-chain molecules;

(c) re-associating said single-chain molecules;

(d) separating multivalent antigen-binding proteins from said single-chain molecules; and

(e) recovering said multivalent proteins.

23. The method of claim 22 wherein said dissociation is caused by dialysis against a dissociating solution.

24. The method of claim 22 wherein said reassociation is caused by dialysis against a refolding solution or a refolding agent.

25. A method of producing a multivalent antigen-binding protein, comprising the step of cross-linking at least two single-chain molecules to each other, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

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(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule.

26. The method of claim 25 wherein said cross-linking is effected by chemical means.

27. A method of producing a multivalent antigen-binding protein, comprising the steps of:

(a) producing a composition comprising single-chain molecules, each single-chain molecule comprising:

(i) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(ii) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(iii) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule;

(b) concentrating said single-chain molecules;

(c) separating said multivalent protein from said single-chain molecules; and

(d) recovering said multivalent protein.

28. The method of claim 27 wherein said concentrating step occurs from approximately 0.5 mg/ml single-chain molecule to the concentration at which precipitation starts.

29. A method of detecting an antigen in or suspected of being in a sample, which comprises:

(a) contacting said sample with the multivalent antigen-binding protein of claim 1; and

(b) detecting whether said multivalent antigen-binding protein has bound to said antigen.

30. A method of imaging the internal structure of an animal, comprising administering to said animal an effective amount of a labeled form of the multivalent antigen-binding protein of claim 1 and measuring detectable radiation associated with said animal.

31. A composition comprising an association of a multivalent antigen-binding protein as claimed in any one of claims 1-4, 8-11, or 15-18 with a therapeutically or diagnostically effective agent.

32. A single-chain protein comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody light chain;

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

33. A single-chain protein comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

34. A single-chain protein comprising:

(a) a first polypeptide comprising the V_L or V_H of a CC49 monoclonal antibody;

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(b) a second polypeptide comprising the V_L or V_H of a CC49 monoclonal antibody; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

35. The single-chain protein of claim 34 wherein said linker is selected from the group consisting of the 202', 212, 216, and 217 linkers.

36. A single-chain protein comprising:

(a) a first polypeptide comprising the V_L or V_H of a CC49 monoclonal antibody;

(b) a second polypeptide comprising the V_L or V_H of a 4-4-20 monoclonal antibody; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

37. The single-chain protein of claim 36 wherein said linker is selected from the group consisting of the 202', 212, 216, and 217 linkers.

38. A genetic sequence which codes for the single-chain protein of claim 32, comprising:

(a) a DNA sequence coding for a first polypeptide comprising the binding portion of the variable region of an antibody light chain;

(b) a DNA sequence coding for a second polypeptide comprising the binding portion of the variable region of an antibody light chain;

(c) a DNA sequence coding for a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

39. A genetic sequence which codes for the single-chain protein of claim 33, comprising:

(a) a DNA sequence coding for a first polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(b) a DNA sequence coding for a second polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(c) a DNA sequence coding for a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

40. A genetic sequence which codes for the single-chain protein of claim 34, comprising:

(a) a DNA sequence coding for the V_L or V_H of a CC49 monoclonal antibody;

(b) a DNA sequence coding for the V_L or V_H of a CC49 monoclonal antibody;

(c) a DNA sequence coding for a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

41. The genetic sequence of claim 40 wherein said DNA sequence codes for a peptide linker selected from the group consisting of the 202', 212, 216, and 217 linkers.

42. A genetic sequence which codes for the single-chain protein of claim 36, comprising:

(a) a DNA sequence coding for the V_L or V_H of a CC49 monoclonal antibody;

(b) a DNA sequence coding for the V_L or V_H of a 4-4-20 monoclonal antibody;

(c) a DNA sequence coding for a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain protein.

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43. The genetic sequence of claim 42 wherein said DNA sequence codes for a peptide linker selected from the group consisting of the 202', 212, 216, and 217 linkers.

44. A multivalent single-chain antigen-binding protein comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein;

(d) a third polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(e) a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(f) a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and

(g) a peptide linker linking said second and third polypeptides (b) and (d) into said multivalent protein.

45. A multivalent single-chain antigen-binding protein comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein;

(d) a third polypeptide comprising the binding portion of the variable region of an antibody light chain;

(e) a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy chain;

(f) a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and

(g) a peptide linker linking said second and third polypeptides (b) and (d) into said multivalent protein.

46. A genetic sequence which codes for the multivalent antigen-binding protein of claim 44 or 45, comprising:

(a) a DNA sequence coding for a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a DNA sequence coding for a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(c) a DNA sequence coding for a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein

(d) a DNA sequence coding for a third polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(e) a DNA sequence coding for a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(f) a DNA sequence coding for a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and

(g) a DNA sequence coding for a peptide linker linking said second and third polypeptides (b) and (d) into said multivalent protein.

47. A replicable cloning or expression vehicle comprising the DNA sequence of any one of claims 38-43.

48. The vehicle of claim 47 which is a plasmid.

49. A host cell transformed with the vehicle of claim 47.

50. The host cell of claim 49 which is a bacterial cell, a yeast cell or other fungal cell, or a mammalian cell line.

51. A method of producing a multivalent antigen-binding protein comprising two or more single-chain molecules, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain; and

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said single-chain molecule, said method comprising:

(i) providing a genetic sequence coding for said single-chain molecule;

(ii) transforming one or more host cells with said sequence;

(iii) expressing said sequence in said host or hosts; and

(iv) recovering a multivalent protein from said host or hosts.

52. A method of producing a multivalent single-chain antigen-binding protein comprising two or more single-chain molecules, each single-chain molecule comprising:

(a) a first polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(b) a second polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(c) a peptide linker linking said first and second polypeptides (a) and (b) into said multivalent protein;

(d) a third polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

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(e) a fourth polypeptide comprising the binding portion of the variable region of an antibody heavy or light chain;

(f) a peptide linker linking said third and fourth polypeptides (d) and (e) into said multivalent protein; and

(g) a peptide linker linking said second and third polypeptides (b) and (d) into said multivalent protein, said method comprising:

(i) providing a genetic sequence coding for said single-chain molecule;

(ii) transforming one or more host cells with said sequence;

(iii) expressing said sequence in said host or hosts; and

(iv) recovering a multivalent protein from said host or hosts.

53. The method of claim 51 or 52 wherein recovering said multivalent protein comprises separating said multivalent protein from said single-chain molecules.

54. The method of claim 51 or 52 wherein recovering said multivalent protein comprises:

(a) dissociating said single-chain molecules;

(b) re-associating said single-chain molecules;

(c) separating multivalent antigen-binding proteins from said single-chain molecules; and

(d) recovering said multivalent proteins.

55. The method of claim 51 or 52 which further comprises purifying said recovered multivalent protein.

56. The method of claim 51 or 52 wherein said host cell is a bacterial cell, a yeast cell or other fungal cell, or a mammalian cell line.

57. The method of claim 56 wherein said host cell is *E. coli* or *Bacillus subtilis*.

58. The multivalent antigen-binding protein of claim 1 in detectably-labelled form.

59. In an immunoassay method which utilizes an antibody in detectably-labelled form, the improvement comprising using the multivalent protein of claim 58 instead of said antibody.

60. The immunoassay of claim 59 wherein said immunoassay is a competitive immunoassay.

61. The immunoassay of claim 59 wherein said immunoassay is a sandwich immunoassay.

62. In an immunotherapeutic method which utilizes an antibody conjugated to a therapeutic agent, the improvement comprising using the multivalent protein of claim 1 instead of said antibody.

63. In a method of immunoaffinity purification which utilizes an antibody therefor, the improvement which comprises using the molecule of claim 1 instead of said antibody.

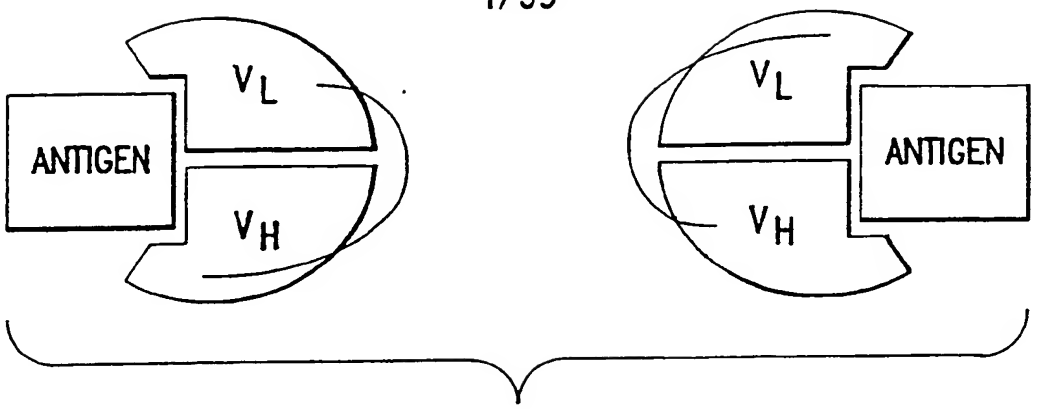


FIG.1A

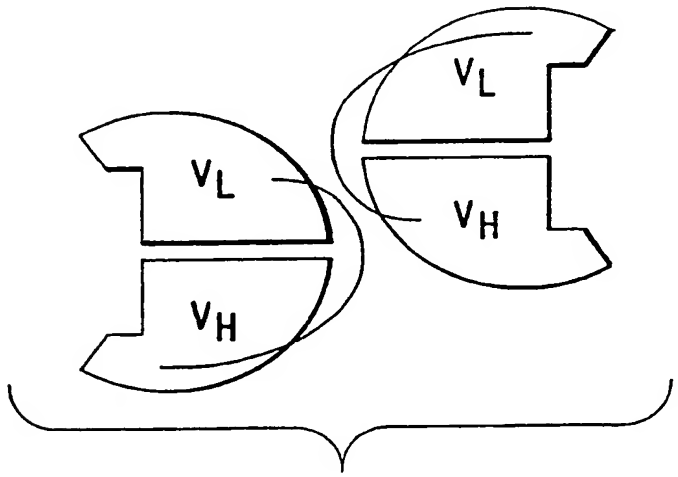


FIG.1B

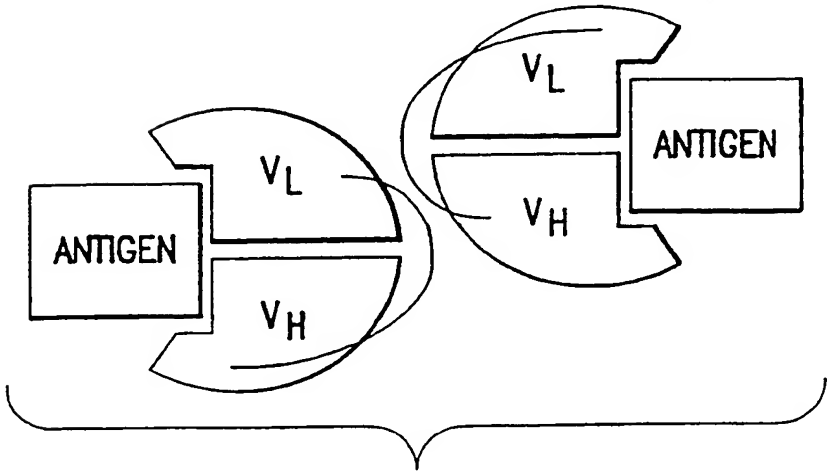


FIG.1C

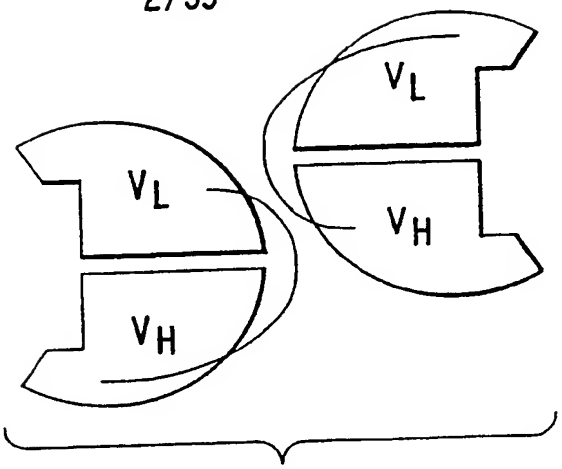


FIG.2A

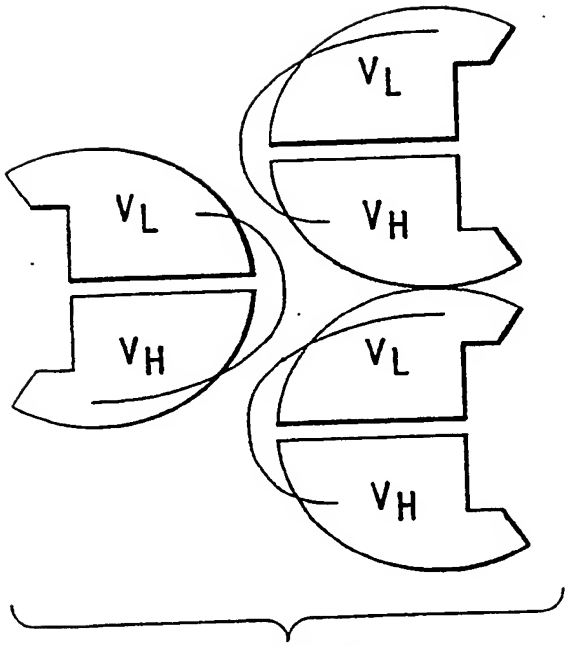


FIG.2B

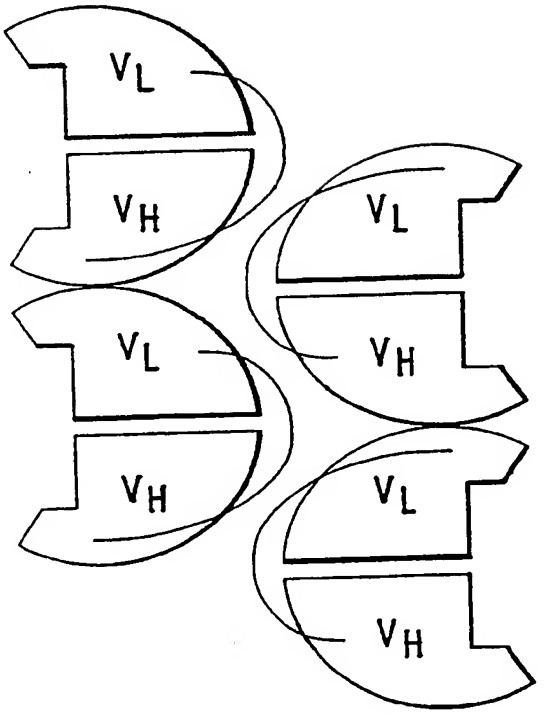


FIG.2C

SDOCID: <WO___9311161A1_I_>

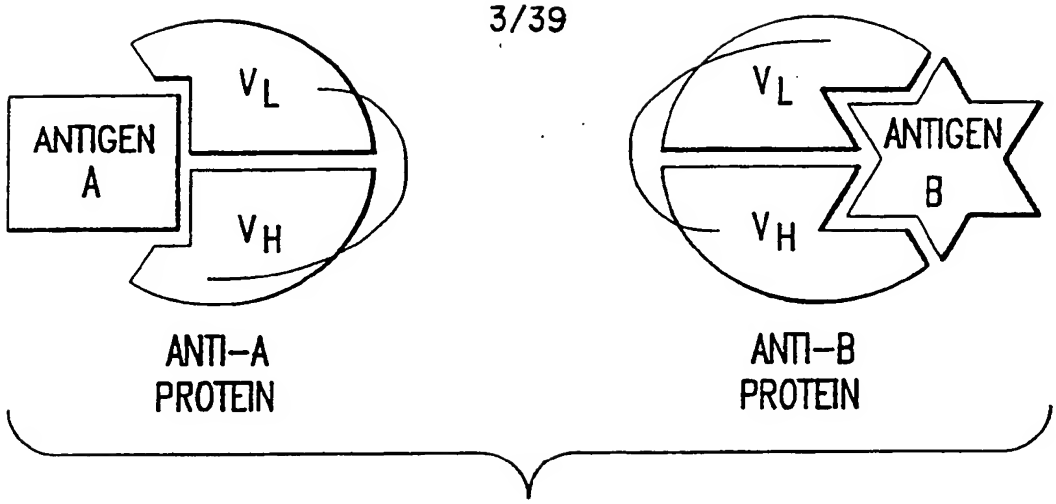


FIG.3A

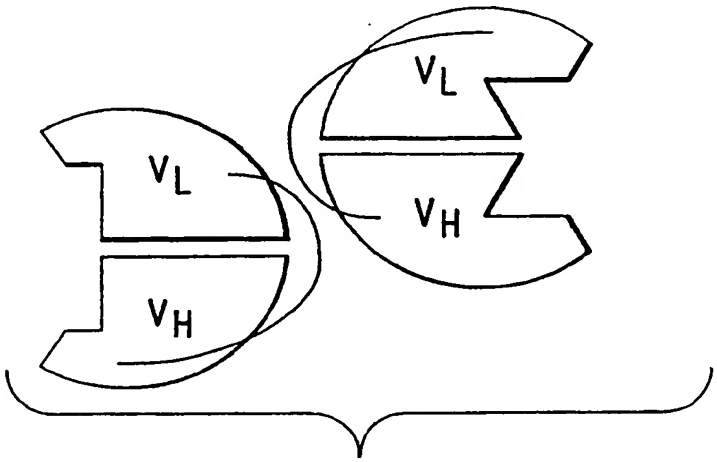


FIG.3B

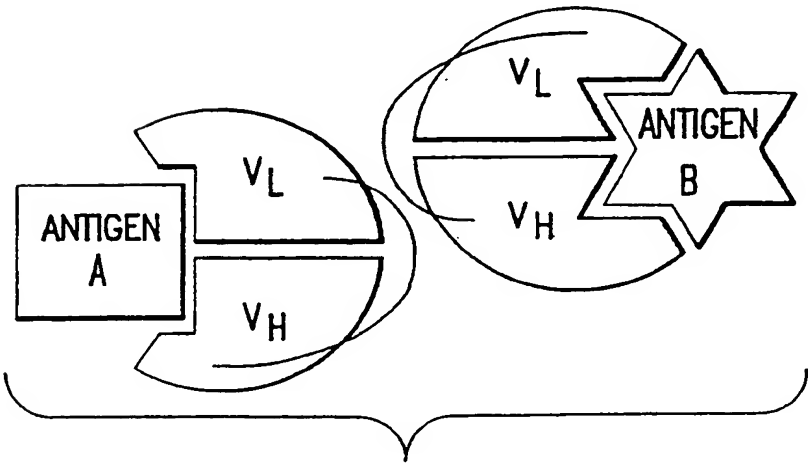


FIG.3C

)OCID: <WO 9311161A1 I >

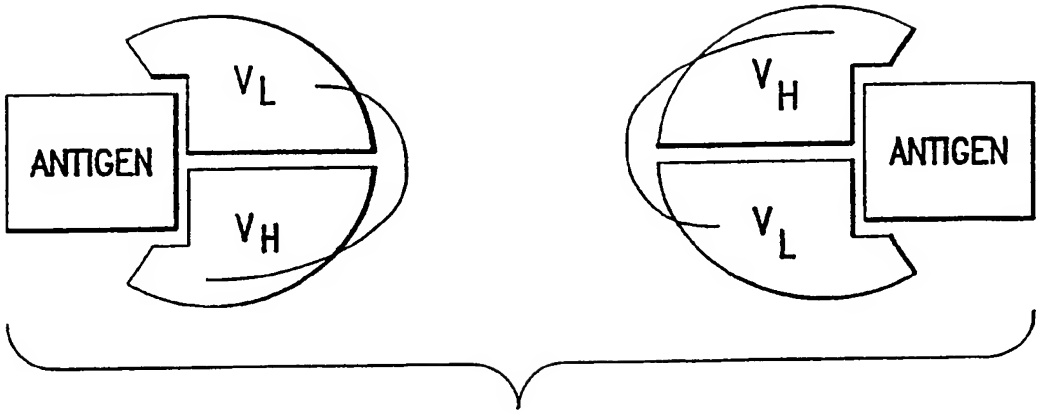


FIG.4A

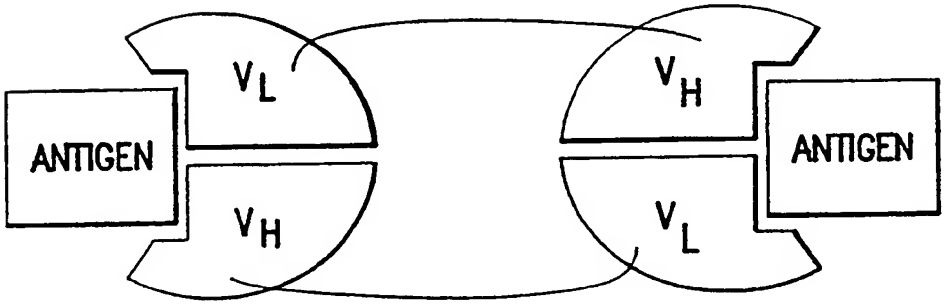


FIG.4B

SDOCID <WO 9311161A1 I >

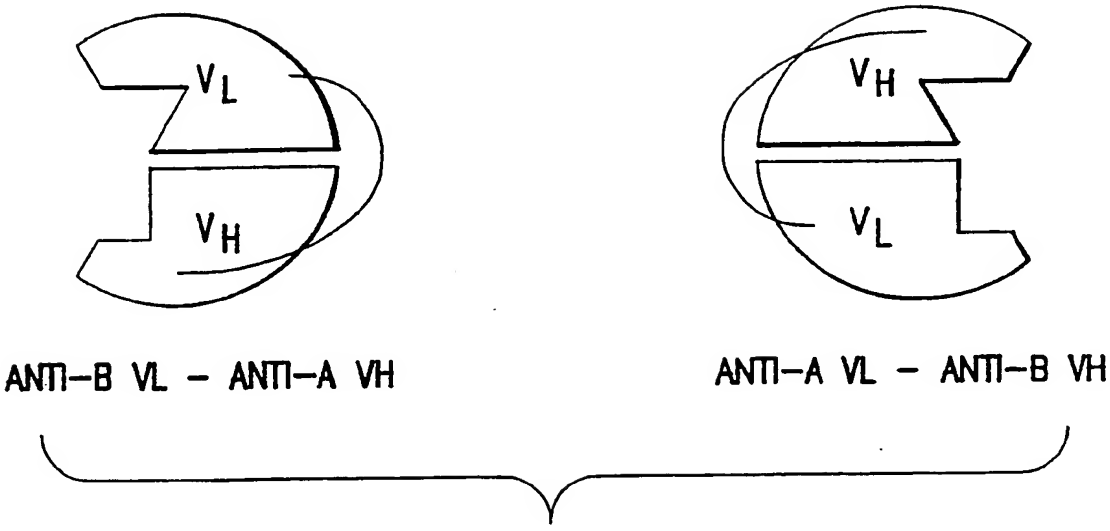


FIG.5A

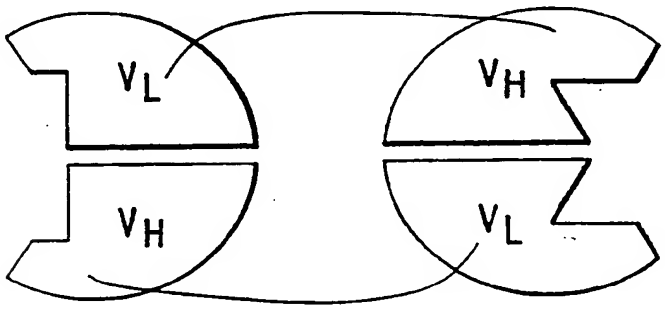


FIG.5B

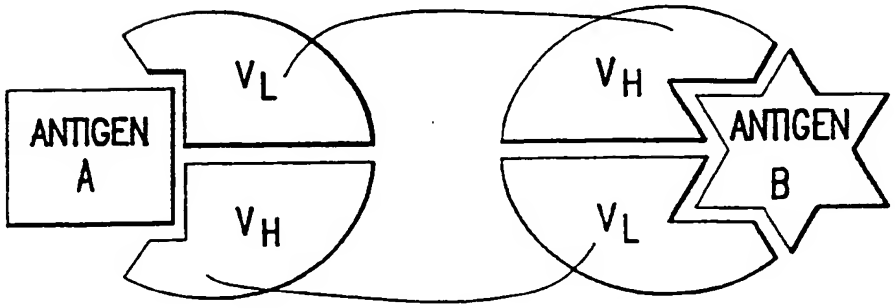


FIG.5C

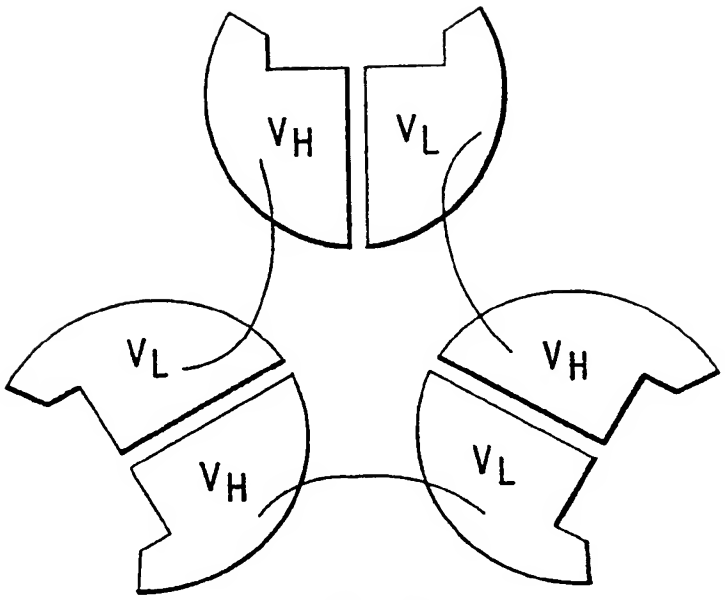


FIG.6A

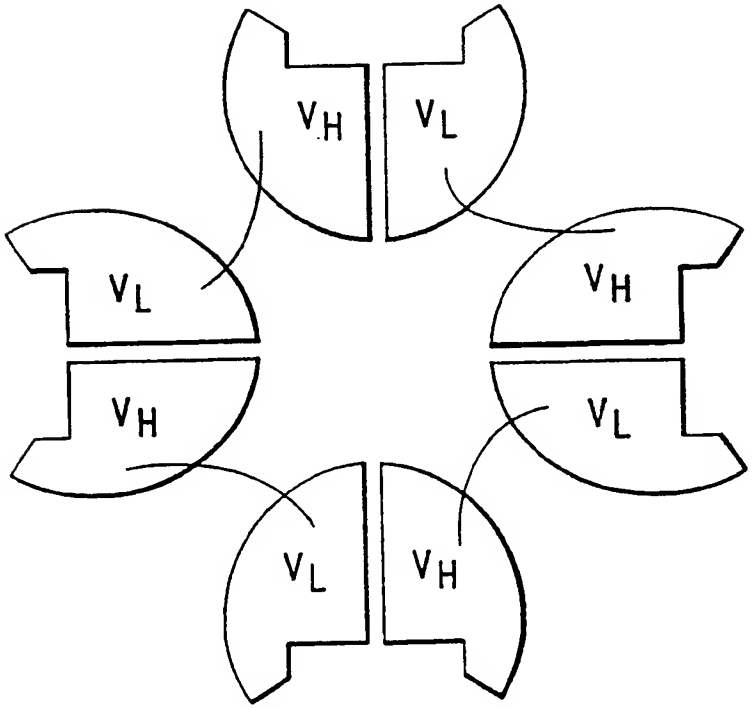


FIG.6B

DOCID: <WO___9311161A1_I_>

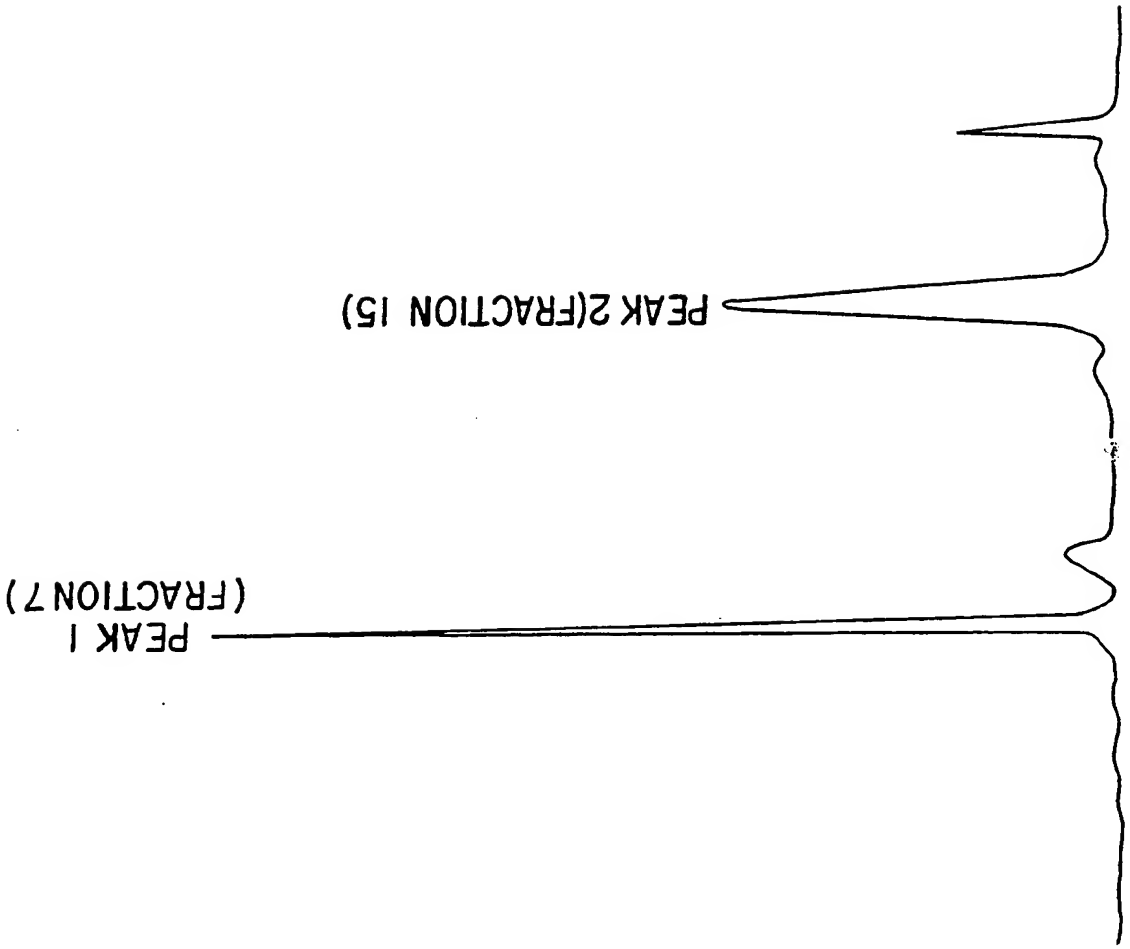


FIG.7

SUBSTITUTE SHEET

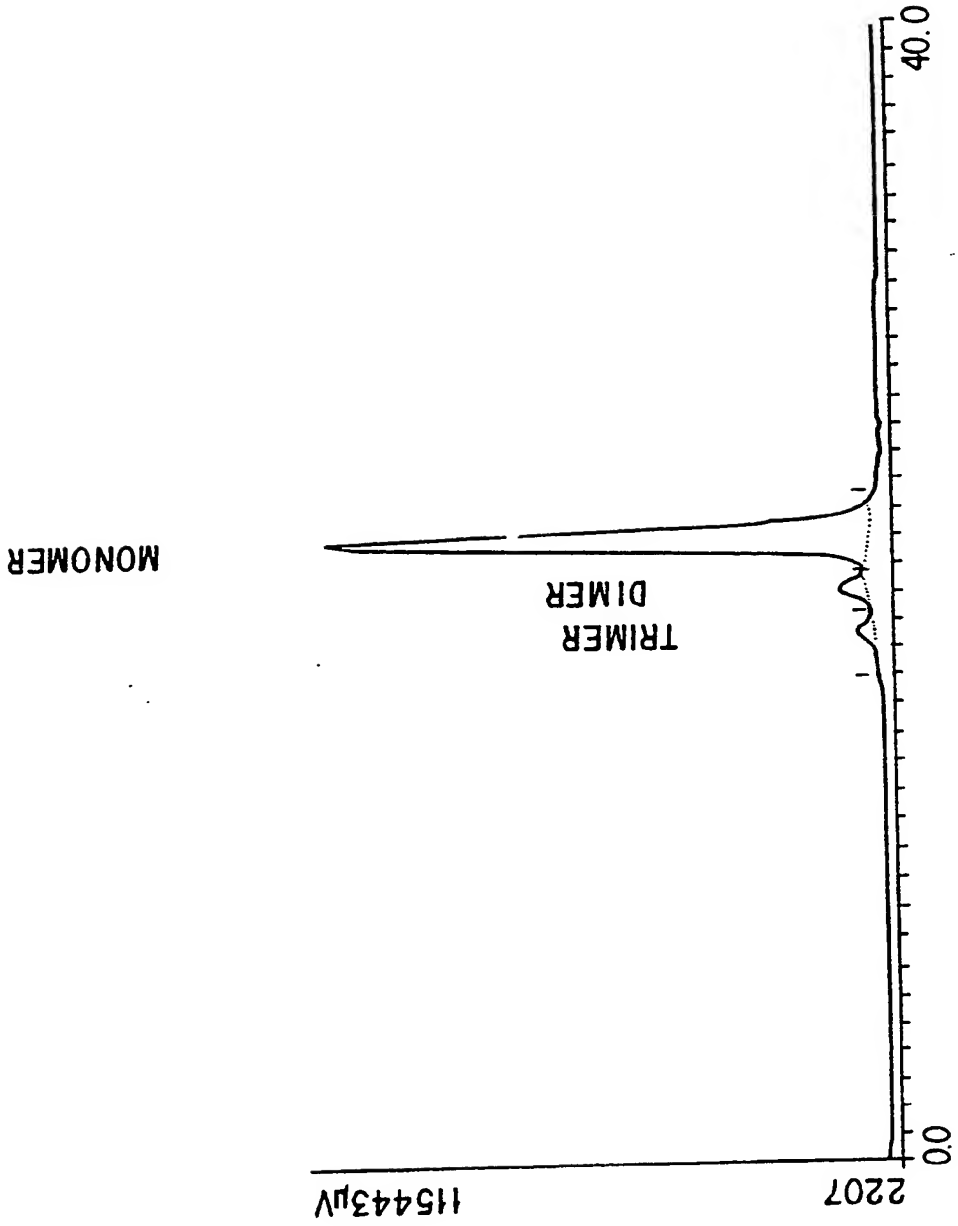


FIG.8

:DOCID: <WO 9311161A1 I >

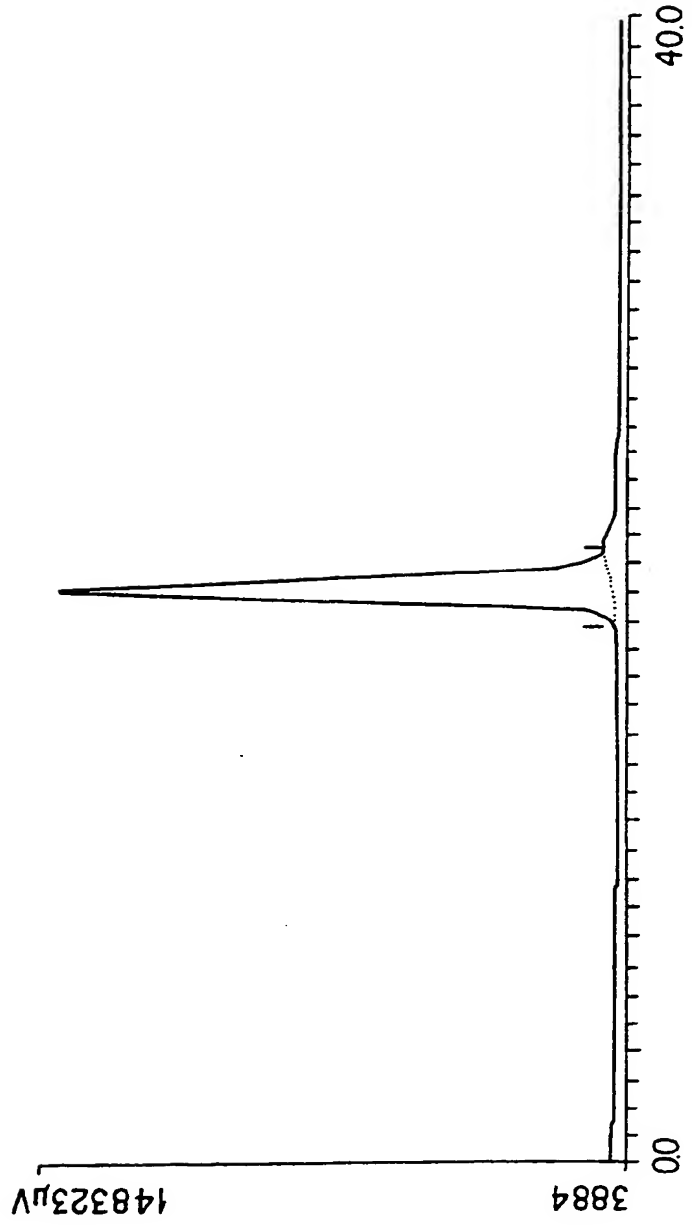


FIG. 9

SUBSTITUTE SHEET

4-4-20 V_L/212/CC49 V_H gene

```
4-4-20 VL                                   10                                  20
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT GAT CAA GCC TCC
Aat II
                                    30                                  40
Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Arg Trp
ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CGT TGG

                                    50                                  60
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe
TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT

                                    70                                  80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC

                                    90                                  100
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCG

                                   110        212 Linker               120
Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Ser Gly Lys
TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA GGT TCT ACC TCT GGT TCT GGT AAA
                        Hind III
                    CC49 VH     130                                 140
Ser Ser Glu Gly Lys Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro
TCC TCT GAA GGC AAA GGT CAG GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT
                            PvuII PstI
                                   150                                 160
Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile
GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA ATT

                                   170                                 180
His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe Ser Pro Gly
CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT TTT TCT CCC GGA
```

FIG.10A

SDOCID: <WO__9311161A1_I_>

1-4-20 V_L/212/CC49 V_H gene

```
                                    190                                 200
Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys
AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC AAA

                                    210                                 220
Ser Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
TCC TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT

                                    230                                 240
TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC
Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser


*** *** Asp
TAA TAG GAT CC
    Bam HI
```

FIG.10A(CONT.)

CC49 V_L/212/4-4-20 V_H gene

CC49 V_L

```
                                                                  10                                                  20
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr
GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT
Aat II
                                                                  30                                                  40
Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC

                                                                  50                                                  60
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG

                                                                  70                                                  80
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC

                                                                  90                                                  100
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT

                                                                  110                 212 Linker                      120
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu Lys Gly Ser Thr Ser Gly Ser Gly
CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG AAA GGC TCT ACT TCC GGT AGC GGC
                                    Hind III
                                4-4-20 VH                                                                             140
Lys Ser Ser Glu Gly Lys Gly Glu Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln
AAA TCT TCT GAA GGT AAA GGT GAA GTT AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA

                                                                  150                                                 160
Pro Gly Arg Pro Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp
CCT GGG AGG CCC ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG

                                                                  170                                                 180
Met Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile Arg Asn
ATG AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA ATT AGA AAC
```

FIG.10B

:DOCID: <WO___9311161A1_I_>

CC49 V_L/212/4-4-20 V_H gene

```
                                          190                                 200
Lys Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys Gly Arg Phe Thr Ile Ser
AAA CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG AAA GGC AGA TTC ACC ATC TCA

                                          210                                 220
Arg Asp Asp Ser Lys Ser Ser Val Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met
AGA GAT GAT TCC AAA AGT AGT GTC TAC CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG

                                          230                                 240
Gly Ile Tyr Tyr Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser
GGT ATC TAT TAC TGT ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCA


Val Thr Val Ser  *   *  Gly Ser
GTC ACC GTC TCC TAA TAA GGA TCC
                        Bam HI
```

FIG.10B(CONT.)

DOCID <WO 9311161A1 I >

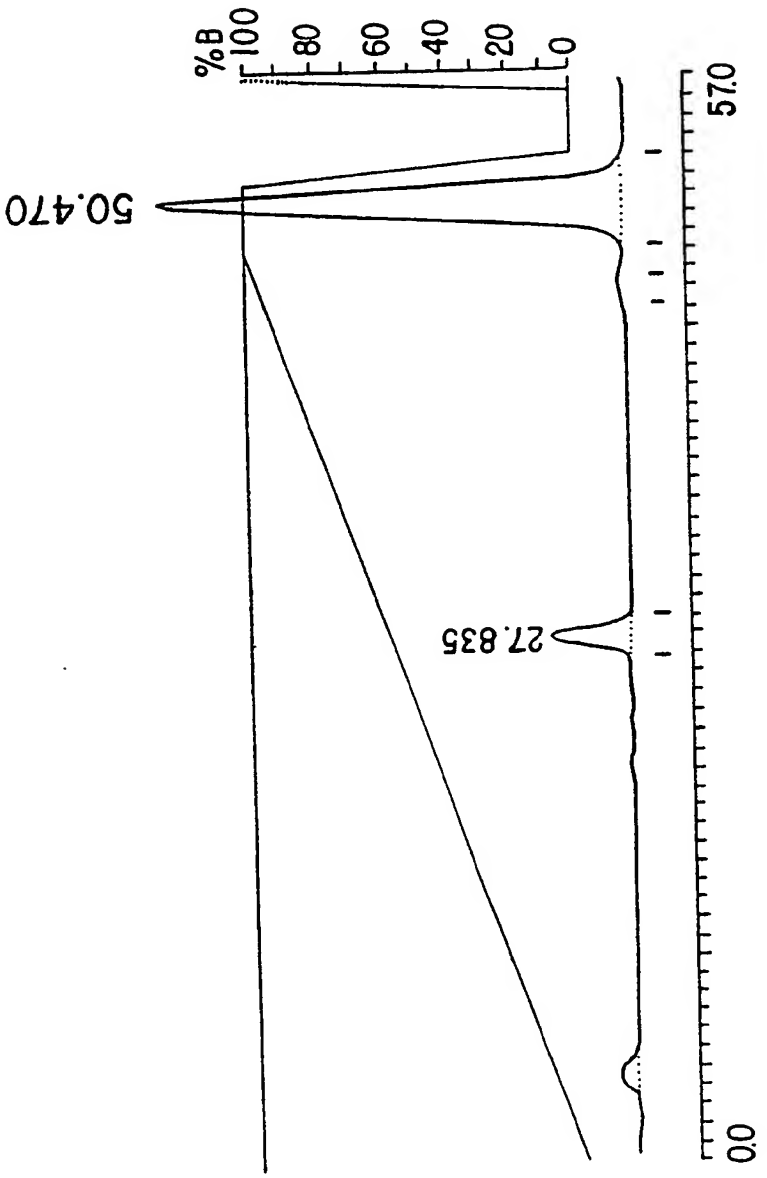


FIG.11

SDOCID: <WO___9311161A1_I_>

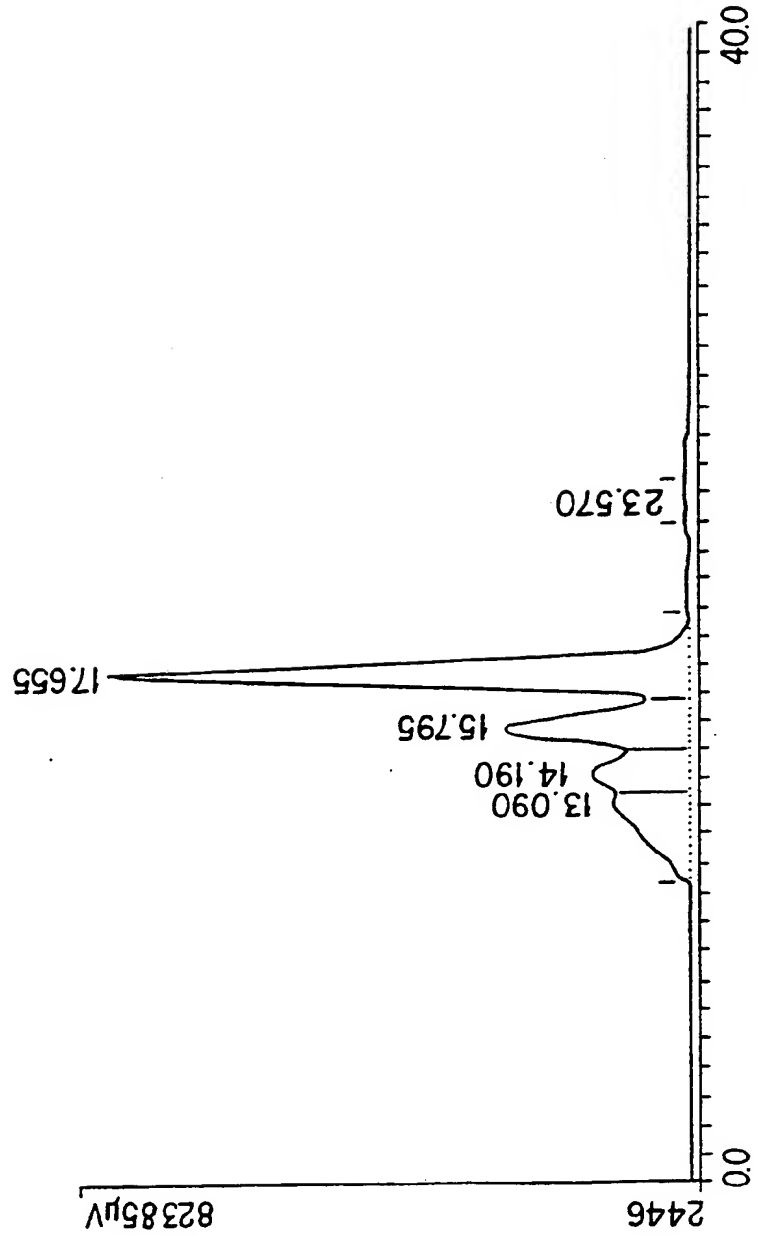


FIG. 12

SUBSTITUTE SHEET

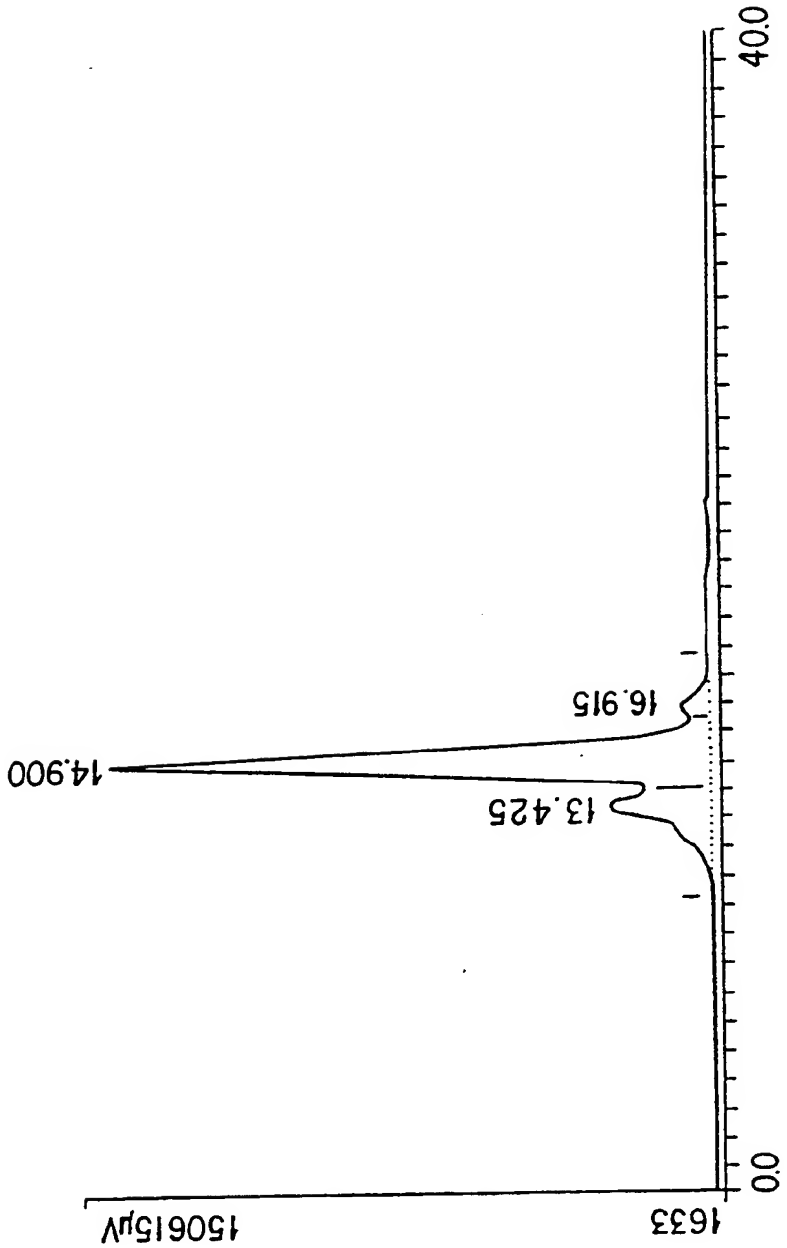


FIG.13

SDOCID. <WO 9311161A1 I >

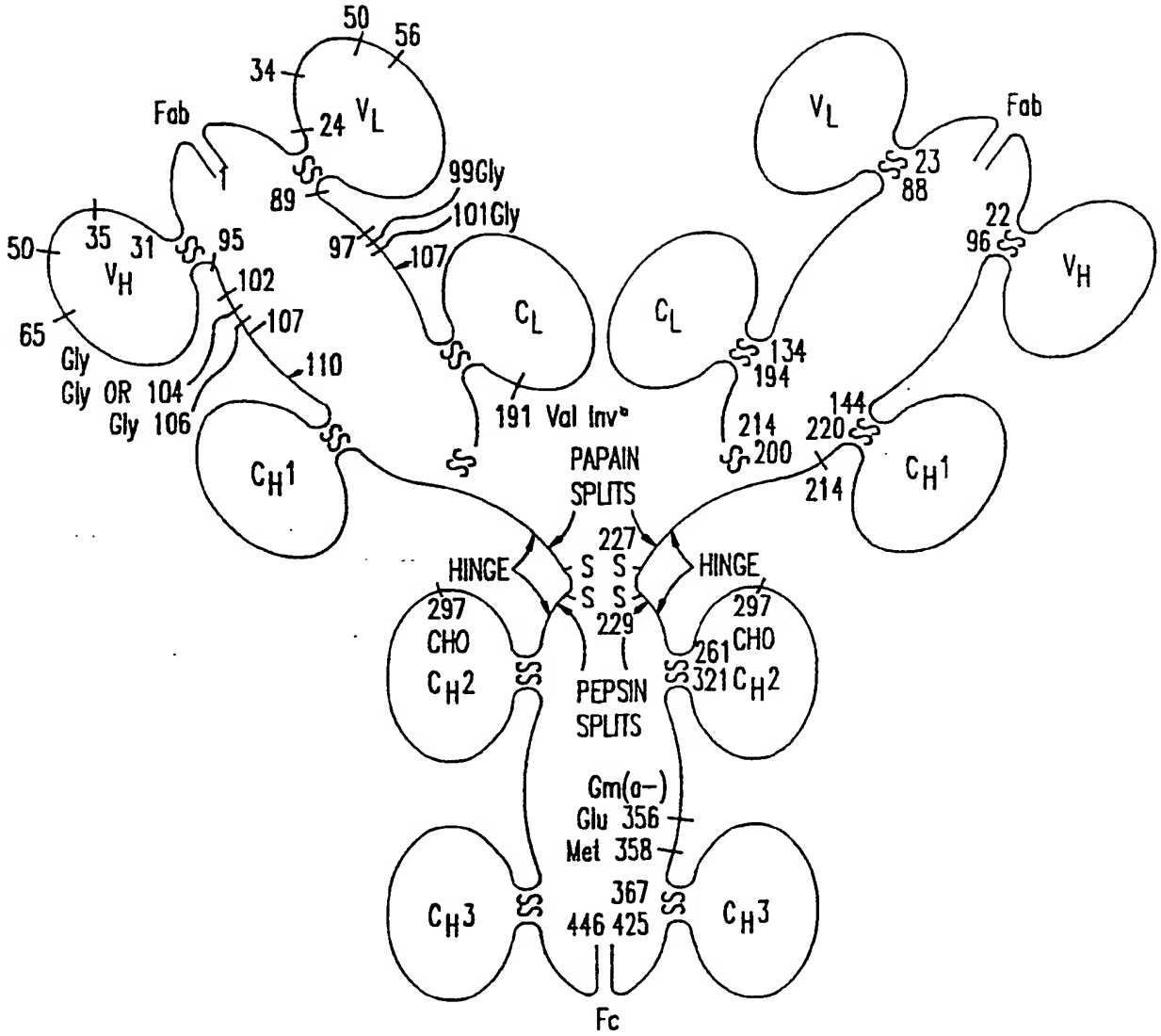


FIG.14

)OCID: <WO___9311161A1_I_>

4-4-20/212 protein with single cysteine hinge

4-4-20 V_L

```
                                                                  10                                                  20
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT GAT CAA GCC TCC
Aat II
                                                                  30                                                  40
Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Arg Trp
ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CGT TGG

                                                                  50                                                  60
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe
TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT

                                                                  70                                                  80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC

                                                                  90                                                  100
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCG

                                                                  110          212 Linker                             120
Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Ser Gly Lys
TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA GGT TCT ACC TCT GGT TCT GGT AAA
                                Hind III
                                4-4-20 VH                                                                             140
Ser Ser Glu Gly Lys Gly Glu Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro
TCT TCT GAA GGT AAA GGT GAA GTT AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT

                                                                  150                                                 160
Gly Arg Pro Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
GGG AGG CCC ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG

                                                                  170                                                 180
Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile Arg Asn Lys
AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA ATT AGA AAC AAA
```

FIG.15A

SDOCID: <WO___9311161A1_I_>

4-4-20/212 protein with single cysteine hinge

```
                                    190                                 200
Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg
CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG AAA GGC AGA TTC ACC ATC TCA AGA

                                    210                                 220
Asp Asp Ser Lys Ser Ser Val Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly
GAT GAT TCC AAA AGT AGT GTC TAC CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT

                                    230                                 240
Ile Tyr Tyr Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
ATC TAT TAC TGT ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCG GTC
                                                                    Bst EII

          Hinge                     250
Thr Val Ser Ser Asp Lys Thr His Thr Cys *** ***
ACC GTC TCC AGT GAT AAG ACC CAT ACA TGC TAA TAG GAT CC
                                              Bam HI
```

pGx 5532, Gx 8932

FIG.15A(CONT.)

XOCID: <WO__9311161A1_I_>

4-4-20/212 protein with two cysteine hinge

4-4-20 V_L

```
                                    10                                  20
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT GAT CAA GCC TCC
Aat II
                                    30                                  40
Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Arg Trp
ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CGT TGG

                                    50                                  60
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe
TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT

                                    70                                  80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC

                                    90                                  100
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCG

                                    110        212 Linker               120
Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Ser Gly Lys
TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA GGT TCT ACC TCT GGT TCT GGT AAA
                           Hind III
                        4-4-20 V_H  130                                 140
Ser Ser Glu Gly Lys Gly Glu Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro
TCT TCT GAA GGT AAA GGT GAA GTT AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT

                                    150                                 160
Gly Arg Pro Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met
GGG AGG CCC ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG

                                    170                                 180
Asn Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile Arg Asn Lys
AAC TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA ATT AGA AAC AAA
```

FIG.15B

DOCID: <WO___9311161A1_I_>

4-4-20/212 protein with two cysteine hinge

```
                                    190                                 200
Pro Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg
CCT TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG AAA GGC AGA TTC ACC ATC TCA AGA

                                    210                                 220
Asp Asp Ser Lys Ser Ser Val Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly
GAT GAT TCC AAA AGT AGT GTC TAC CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT

                                    230                                 240
Ile Tyr Tyr Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
ATC TAT TAC TGT ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCG GTC
                                                                    Bst EII

          Hinge                     250
Thr Val Ser Ser Asp Lys Thr His Thr Cys Pro Pro Cys *** ***
ACC GTC TCC AGT GAT AAG ACC CAT ACA TGC CCT CCA TGC TAA TAG GAT CC
                                                        Bam H1
```

pGx 5533, Gx 8933

FIG.15B(CONT.)

CC49/212 SCA™ protein genetic dimer

CC49 V_L

```
                                                                  10                                                  20
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr
GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT
Aat II
                                                                  30                                                  40
Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC

                                                                  50                                                  60
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG

                                                                  70                                                  80
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC

                                                                  90                                                  100
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT

                                                                  110                 212 Linker                      120
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu Lys Gly Ser Thr Ser Gly Ser Gly
CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG AAA GGC TCT ACT TCC GGT AGC GGC
                                    Hind III
                                CC49 V_H                                                                            140
Lys Ser Ser Glu Gly Lys Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys
AAA TCC TCT GAA GGC AAA GGT CAG GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA
                                    PvuII PstI
                                150                                                                                 160
Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala
CCT GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA

                                                                  170                                                 180
Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe Ser Pro
ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT TTT TCT CCC
```

FIG.16A

CC49/212 SCA^{TM} protein genetic dimer

```
                                                190                                                 200
Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp
GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG GGC AAG GCC ACA CTG ACT GCA GAC

                                                210                                                 220
Lys Ser Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val
AAA TCC TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT GCA GTG

                                                230                                                 240
Tyr Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val Thr Val
TAT TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC ACC GTC

        CC49 VL                                 250                                                 260
Ser Ser Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly Glu Lys
TCC TCA GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG
        Aat II
                                                270                                                 280
Val Thr Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr
GTT ACT TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC

                                                290                                                 300
Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser
TTG GCC TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC

                                                310                                                 320
Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr
GCT AGG GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT

                                                330                                                 340
Leu Ser Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr
CTC TCC ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT

                                                350                                 212 Linker      360
Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu Lys Gly Ser Thr Ser Gly
AGC TAT CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG AAA GGC TCT ACT TCC GGT
                                                Hind III
                                                CC49 VH                                             380
Ser Gly Lys Ser Ser Glu Gly Lys Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu
AGC GGC AAA TCC TCT GAA GGC AAA GGT CAG GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG
                                                PvuII PstI
```

FIG.16B

DOCID: <WO 9311161A1 I >

CC49/212 SCA^{TM} protein genetic dimer

```
                                    390                                 400
Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp
GTG AAA CCT GGG GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC

                                    410                                 420
His Ala Ile His Trp Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe
CAT GCA ATT CAC TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT TTT

                                    430                                 440
Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala Thr Leu Thr
TCT CCC GGA AAT GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG GGC AAG GCC ACA CTG ACT

                                    450                                 460
Ala Asp Lys Ser Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser Leu Thr Ser Glu Asp Ser
GCA GAC AAA TCC TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC CTG ACA TCT GAG GAT TCT

                                    470                                 480
Ala Val Tyr Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Ser Val
GCA GTG TAT TTC TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA ACC TCA GTC


Thr Val Ser *** *** Asp
ACC GTC TCC TAA TAG GAT CC
                Bam H1
```

FIG.16C

SDOCID: <WO___9311161A1_I_>

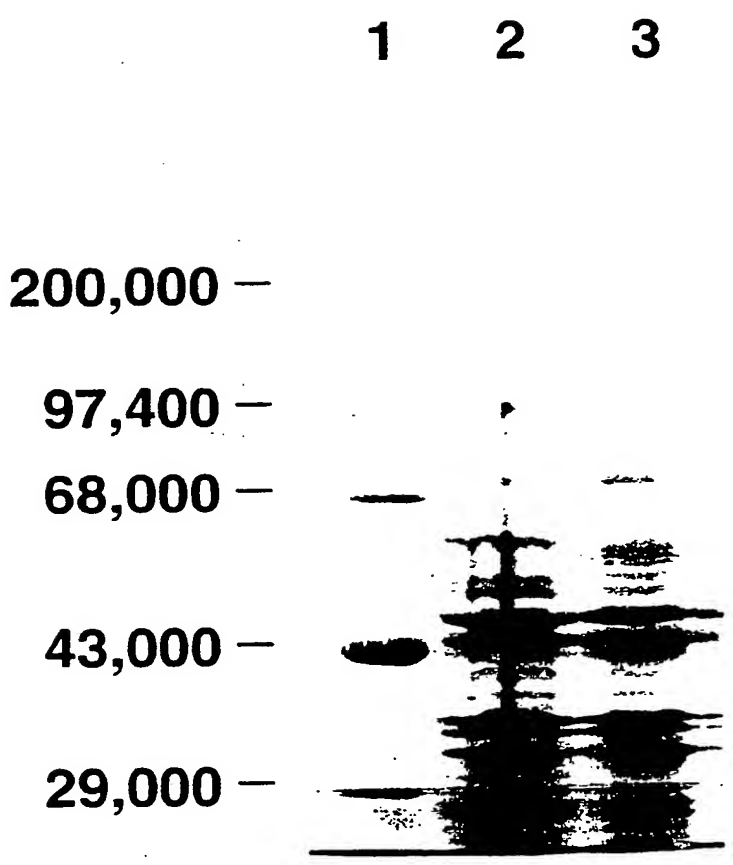


FIG. 17

SUBSTITUTE SHEET

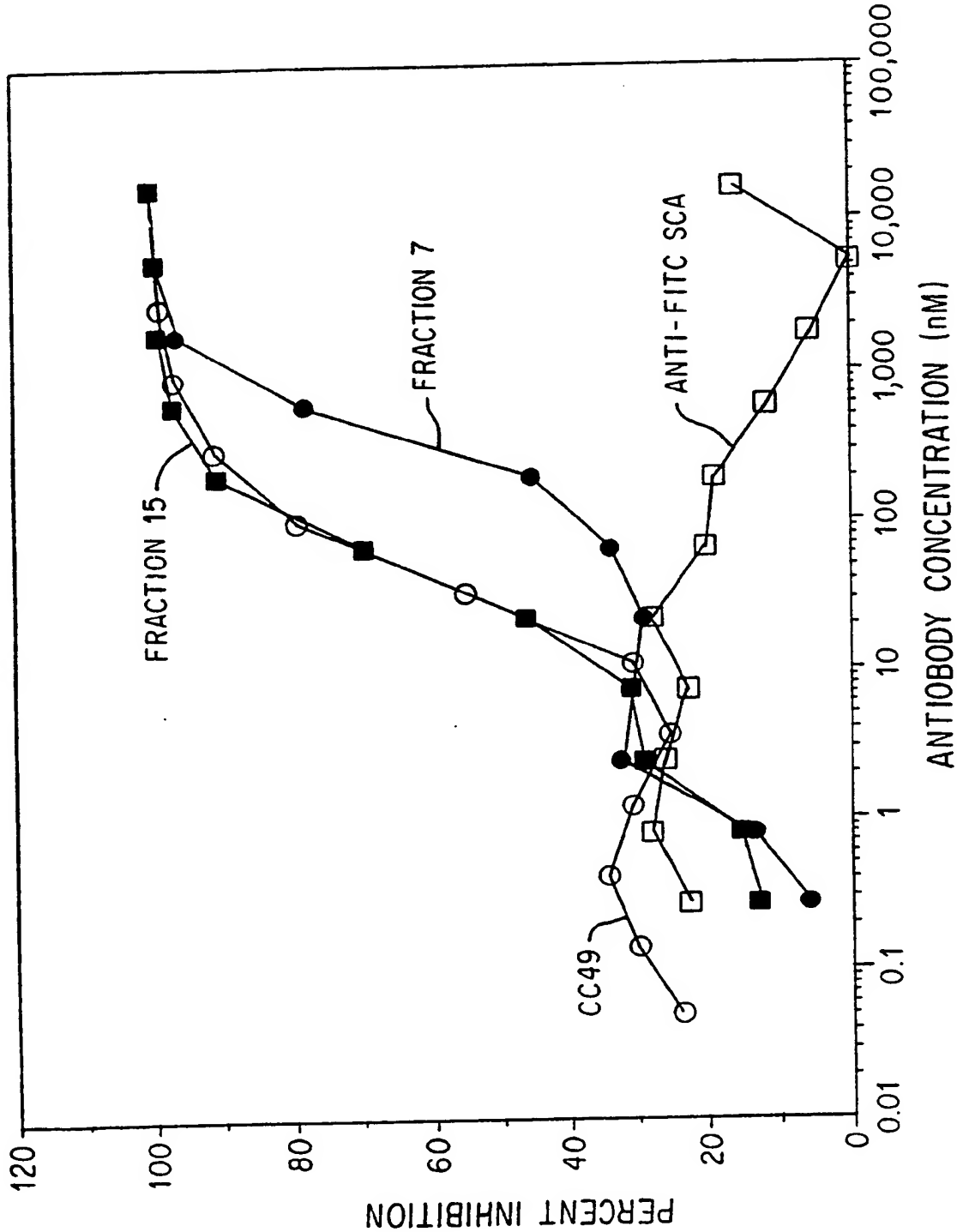


FIG. 18

SDOCID: <WO___9311161A1 I >

4-4-20 V_L/217/CC49 V_H gene

```
4-4-20 VL                                          10                                              20
Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu Gly Asp Gln Ala Ser
GAC GTC GTT ATG ACT CAG ACA CCA CTA TCA CTT CCT GTT AGT CTA GGT GAT CAA GCC TCC
Aat II
                                                   30                                              40
Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Arg Trp
ATC TCT TGC AGA TCT AGT CAG AGC CTT GTA CAC AGT AAT GGA AAC ACC TAT TTA CGT TGG

                                                   50                                              60
Tyr Leu Gln Lys Pro Gly Gln Ser Pro Lys Val Leu Ile Tyr Lys Val Ser Asn Arg Phe
TAC CTG CAG AAG CCA GGC CAG TCT CCA AAG GTC CTG ATC TAC AAA GTT TCC AAC CGA TTT

                                                   70                                              80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile
TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC

                                                   90                                             100
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Ser Gln Ser Thr His Val Pro
AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCG

                                                  110         217 Linker                          120
Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Gly Ser Thr Ser Gly Lys Pro Ser
TGG ACG TTC GGT GGA GGC ACC AAG CTT GAA ATC AAA GGT TCT ACC TCT GGT AAA CCA TCT
                            Hind III
                CC49 VH                           130                                             140
Glu Gly Lys Gly Gln Val Gln Leu Gln Gln Ser Asp Ala Glu Leu Val Lys Pro Gly Ala
GAA GGC AAA GGT CAG GTT CAG CTG CAG CAG TCT GAC GCT GAG TTG GTG AAA CCT GGG GCT
                        PvuII PstI
                                                  150                                             160
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp
TCA GTG AAG ATT TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC TGG

                                                  170                                             180
Val Lys Gln Asn Pro Glu Gln Gly Leu Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp
GTG AAA CAG AAC CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT GAT
```

FIG.19A

DOCID: <WO__9311161A1 I >

4-4-20 V_L/217/CC49 V_H gene

							190								200
Asp	Phe	Lys	Tyr	Asn	Glu	Arg	Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr	Ala	Asp	Lys	Ser	Ser
GAT	TTT	AAA	TAC	AAT	GAG	AGG	TTC	AAG	GGC	AAG	GCC	ACA	CTG	ACT	GCA	GAC	AAA	TCC	TCC
							210								220
Ser	Thr	Ala	Tyr	Val	Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp	Ser	Ala	Val	Tyr	Phe	Cys
AGC	ACT	GCC	TAC	GTG	CAG	CTC	AAC	AGC	CTG	ACA	TCT	GAG	GAT	TCT	GCA	GTG	TAT	TTC	TGT
							230								240
Thr	Arg	Ser	Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln	Gly	Thr	Ser	Val	Thr	Val	Ser	***	***
ACA	AGA	TCC	CTG	AAT	ATG	GCC	TAC	TGG	GGT	CAA	GGA	ACC	TCA	GTC	ACC	GTC	TCC	TAA	TAG

Asp
GAT CC
Bam H1

FIG.19A(CONT.)

;DOCID <WO 9311161A1 I >

CC49 V_L/217/4-4-20 V_H gene

CC49 V_L

```
                                    10                                  20
Asp Val Val Met Ser Gln Ser Pro Ser Ser Leu Pro Val Ser Val Gly Glu Lys Val Thr
GAC GTC GTG ATG TCA CAG TCT CCA TCC TCC CTA CCT GTG TCA GTT GGC GAG AAG GTT ACT
AatII
                                    30                                  40
Leu Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala
TTG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT GGT AAT CAA AAG AAC TAC TTG GCC

                                    50                                  60
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC GCT AGG

                                    70                                  80
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Ser
GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC TCC

                                    90                                  100
Ile Ser Ser Val Lys Thr Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
ATC AGC AGT GTG AAG ACT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAG TAT TAT AGC TAT

                                    110          217 Linker             120
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Val Leu Lys Gly Ser Thr Ser Gly Lys Pro
CCC CTC ACG TTC GGT GCT GGG ACC AAG CTT GTG CTG AAA GGC TCT ACT TCC GGT AAA CCA
                               HindIII
                    4-4-20 VH   130                                 140
Ser Glu Gly Lys Gly Glu Val Lys Leu Asp Glu Thr Gly Gly Gly Leu Val Gln Pro Gly
TCT GAA GGT AAA GGT GAA GTT AAA CTG GAT GAG ACT GGA GGA GGC TTG GTG CAA CCT GGG

                                    150                                 160
Arg Pro Met Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp Tyr Trp Met Asn
AGG CCC ATG AAA CTC TCC TGT GTT GCC TCT GGA TTC ACT TTT AGT GAC TAC TGG ATG AAC

                                    170                                 180
Trp Val Arg Gln Ser Pro Glu Lys Gly Leu Glu Trp Val Ala Gln Ile Arg Asn Lys Pro
TGG GTC CGC CAG TCT CCA GAG AAA GGA CTG GAG TGG GTA GCA CAA ATT AGA AAC AAA CCT
```

FIG.19B

DOCID: <WO___9311161A1_I_>

CC49 V_L/217/4-4-20 V_H gene

```
                                    190                                 200
Tyr Asn Tyr Glu Thr Tyr Tyr Ser Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp
TAT AAT TAT GAA ACA TAT TAT TCA GAT TCT GTG AAA GGC AGA TTC ACC ATC TCA AGA GAT

                                    210                                 220
Asp Ser Lys Ser Ser Val Tyr Leu Gln Met Asn Asn Leu Arg Val Glu Asp Met Gly Ile
GAT TCC AAA AGT AGT GTC TAC CTG CAA ATG AAC AAC TTA AGA GTT GAA GAC ATG GGT ATC

                                    230                                 240
Tyr Tyr Cys Thr Gly Ser Tyr Tyr Gly Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr
TAT TAC TGT ACG GGT TCT TAC TAT GGT ATG GAC TAC TGG GGT CAA GGA ACC TCA GTC ACC

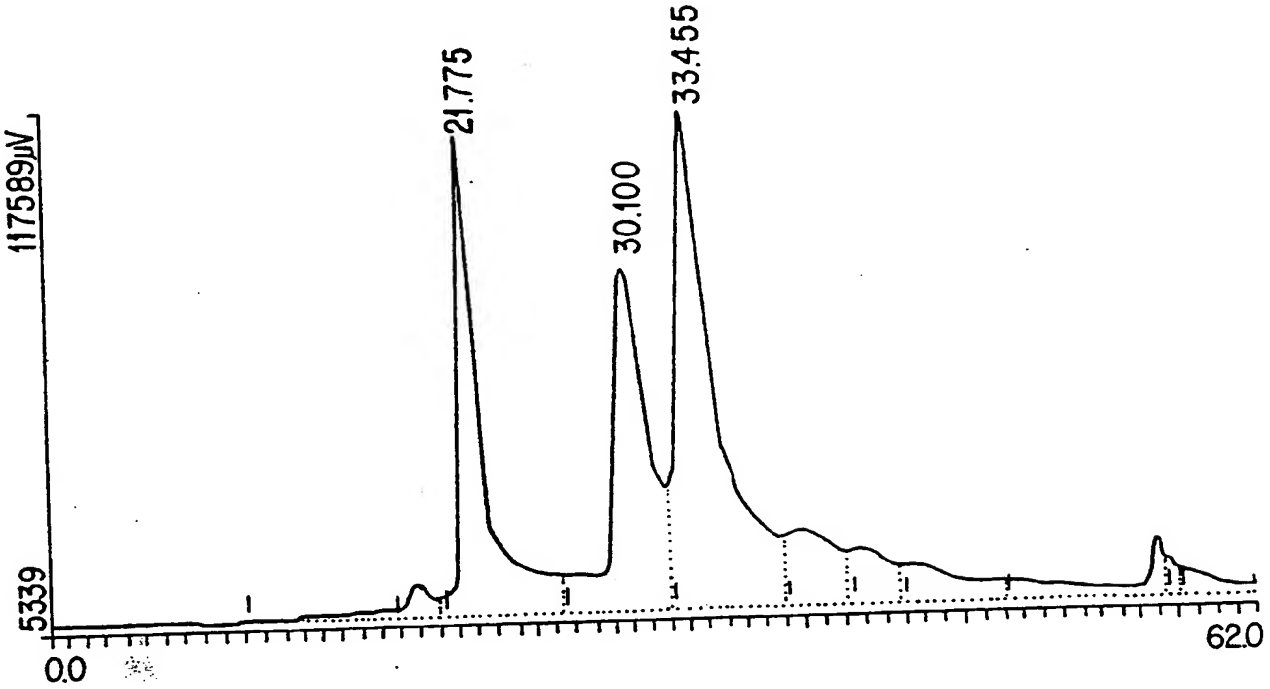

Val Ser *** *** Gly Ser
GTC TCC TAA TAA GGA TCC
                Bam H1
```

FIG.19B(CONT.)

SDOCID: <WO___9311161A1_I_>

PROCESSING FILE: PolyCatA/Proc.CC-49Prep
METHOD: PREP POLY CAT A#2
INJECT VOL: 44
SAMPLING INT: 0.3 SECONDS

CHROMATOGRAM:



ANALYSIS: CHANNEL A

PEAK NO.	TIME	TYPE	HEIGHT(µV)	AREA(µV-SEC)	AREA%
1	17.090	N1	1651	348239	0.778
2	18.940	N2	8014	669441	1.496
3	21.775	N3	104401	8617252	19.263
4	30.100	N4	74925	9753616	21.804
5	33.455	N5	106864	15749605	35.208
6	38.940	N6	17296	2833701	6.334
7	42.010	N7	12645	1637917	3.661
8	44.640	N8	9287	1968584	4.400
9	57.055	N9	13767	2012338	4.498
10	57.610	N10	9323	210914	0.471
11	58.240	X11	6824	930855	2.080
TOTAL AREA				44732462	99.993

FIG.20

DOCID: <WO__9311161A1 I >

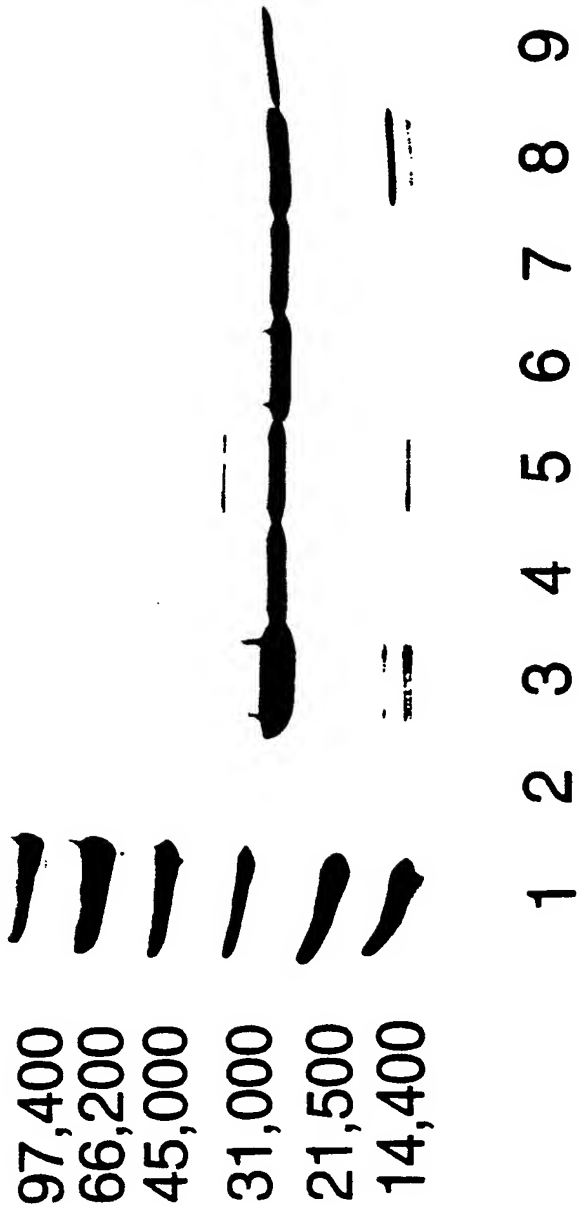
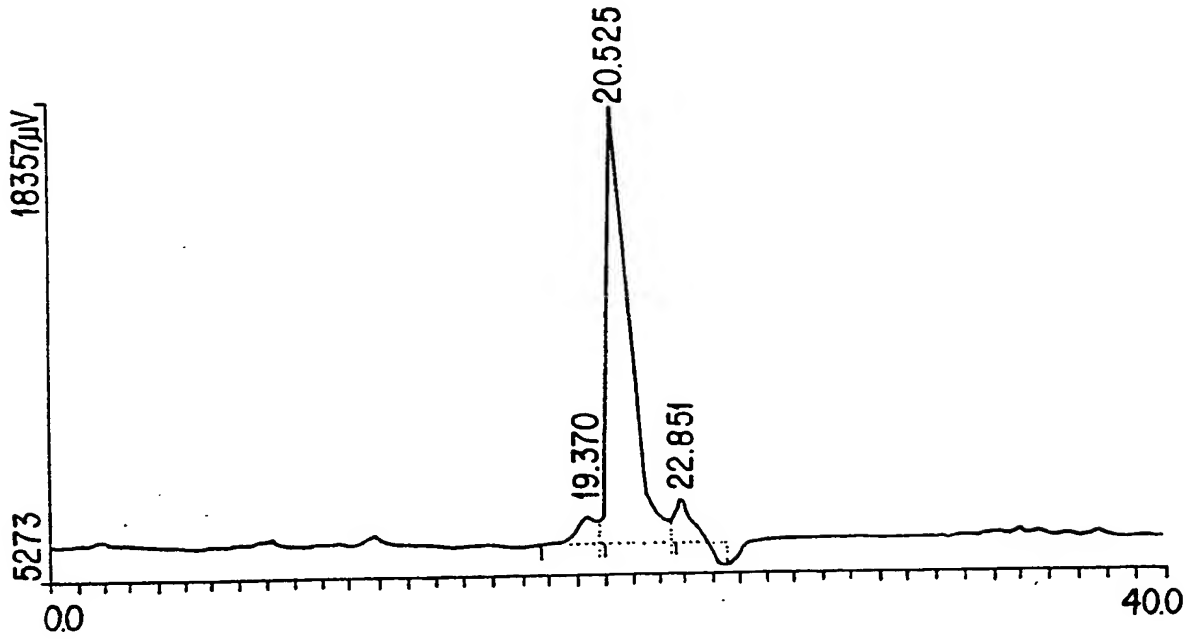


FIG. 21

SDOCID: <WO___9311161A1_I_>

PROCESSING FILE: PolyCatA/Proc.CC-49Prep
METHOD: CC-49 QC SIZE-EXCLUSION
INJECT VOL: .05
SAMPLING INT: 0.1 SECONDS

CHROMATOGRAM:



ANALYSIS: CHANNEL A

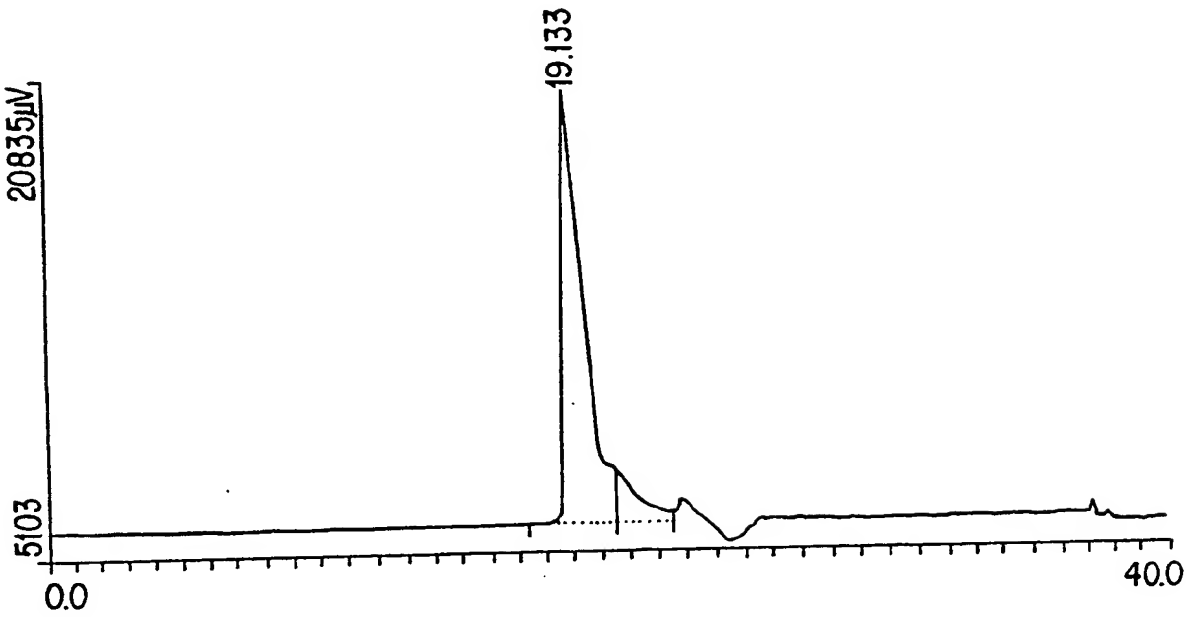
PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-SEC)	AREA%
1	19.370	N1	797	41706	5.694
2	20.525	N2	11789	657280	89.737
3	22.851	N3	1227	33466	4.569
TOTAL AREA				732452	100.000

FIG.22A

DOCID: <WO__9311161A1 I >

PROCESSING FILE: PolyCatA/Proc.CC-49Prep
METHOD: CC-49 QC SIZE-EXCLUSION
INJECT VOL: .05
SAMPLING INT: 0.1 SECONDS

CHROMATOGRAM:



ANALYSIS: CHANNEL A

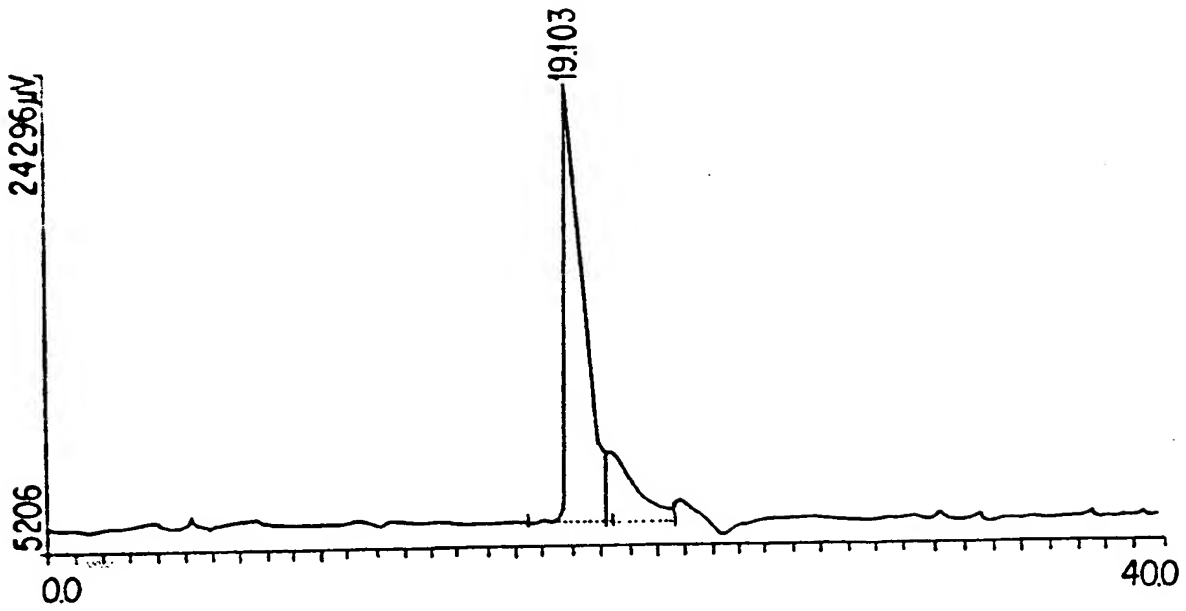
PEAK NO.	TIME	TYPE	HEIGHT(µV)	AREA(µV-SEC)	AREA%
1	19.133	N1	14211	749671	88.214
2	20.538	N2	1863	100154	11.785
TOTAL AREA				849825	99.999

FIG.22B

SDOCID: <WO___9311161A1_I_>

PROCESSING FILE: PolyCatA/Proc.CC-49Prep
METHOD: CC-49 QC SIZE-EXCLUSION
INJECT VOL: .05
SAMPLING INT: 0.1 SECONDS

CHROMATOGRAM:



ANALYSIS: CHANNEL A

PEAK NO.	TIME	TYPE	HEIGHT(μV)	AREA(μV-SEC)	AREA%
1	19.163	N1	17550	876502	83.039
2	20.435	N2	2981	179029	16.961
TOTAL AREA				1055531	100.000

FIG.22C

IOCID: <WO__9311161A1 I >

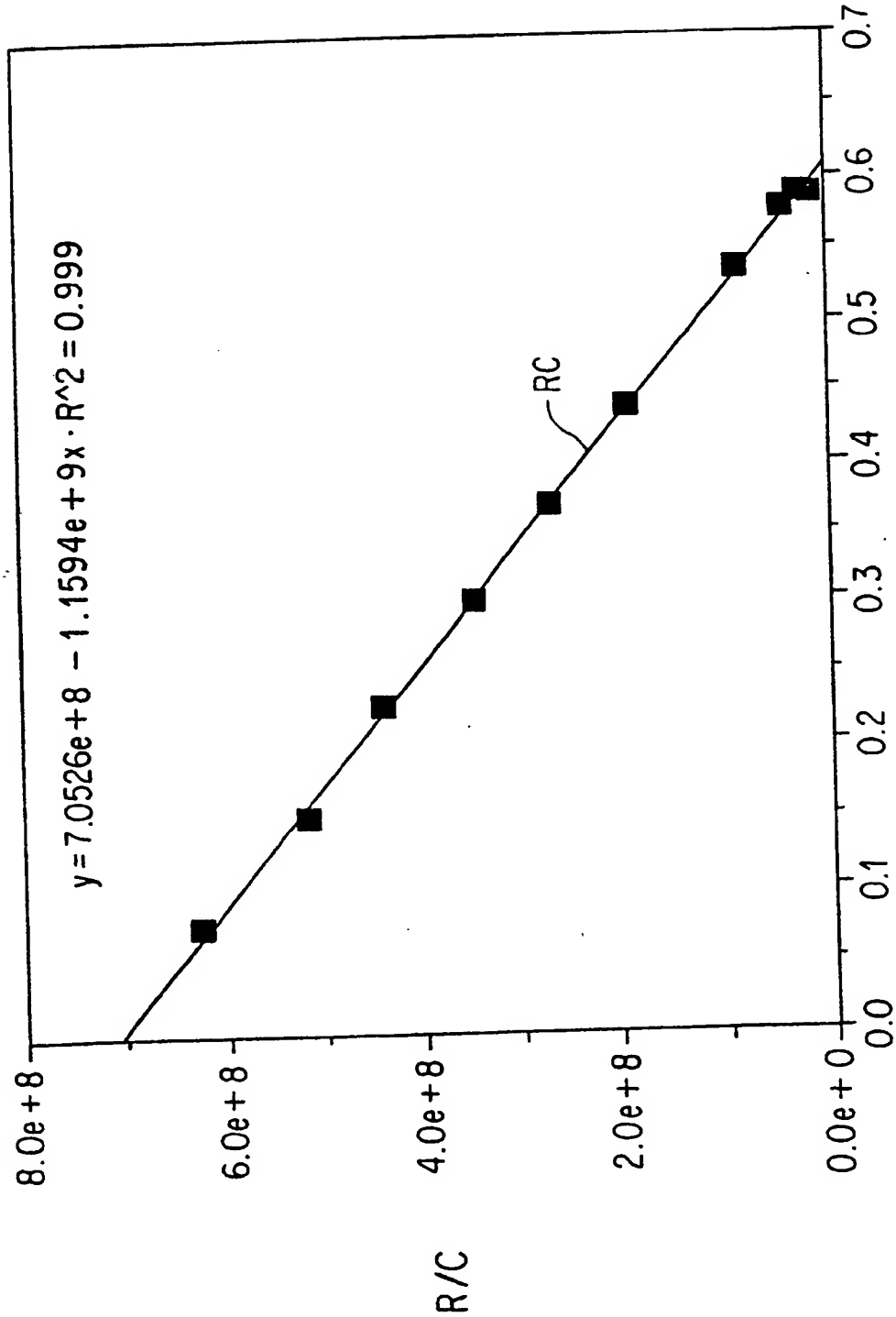


FIG. 23

SDOCID: <WO___9311161A1_I_>

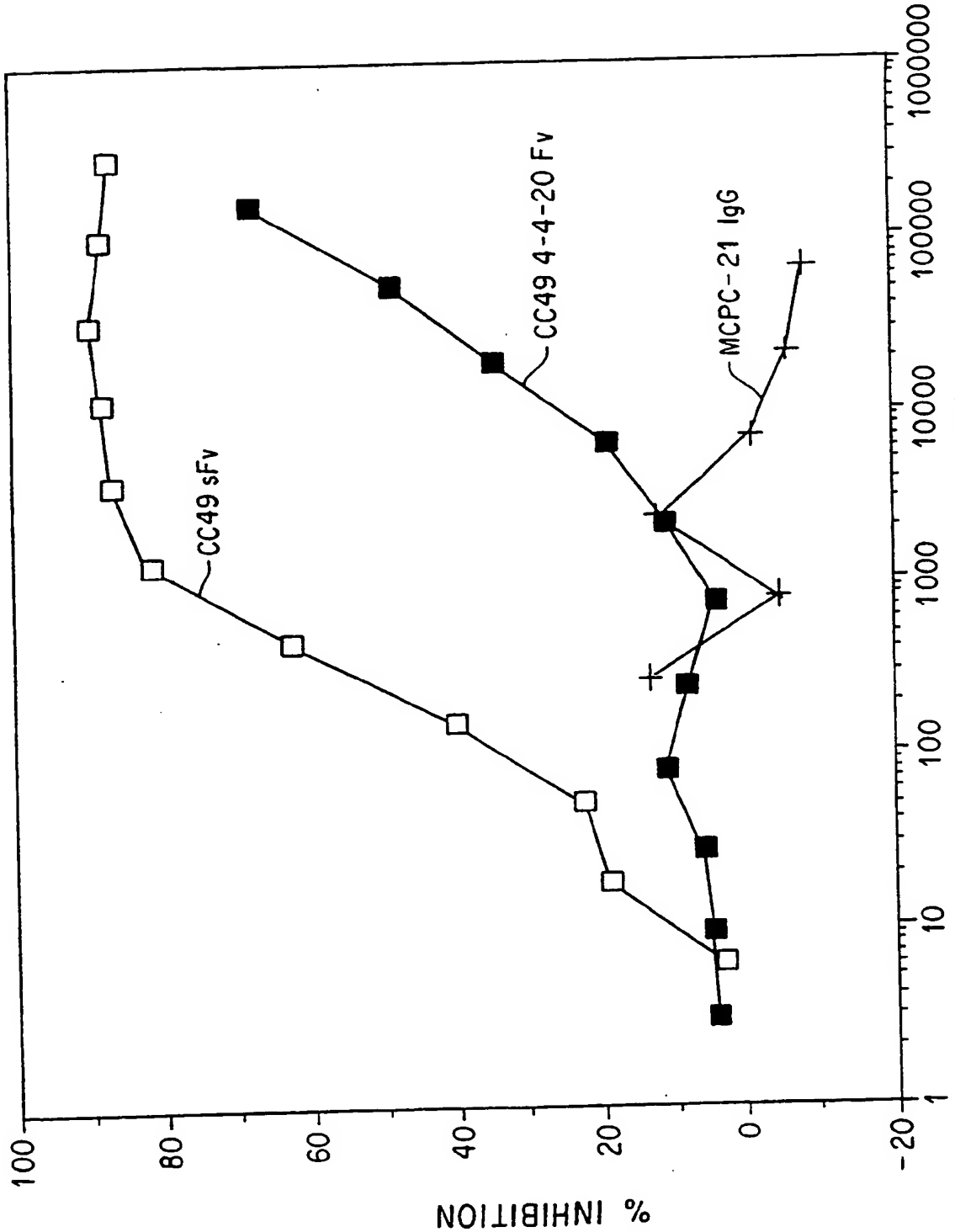


FIG. 24

)OCID: <WO___9311161A1 I >

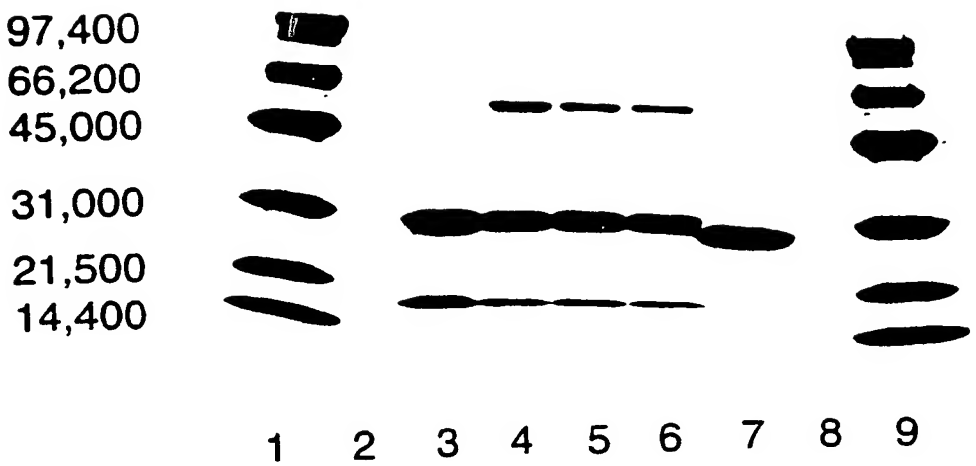


FIG. 25

DOCID: <WO___9311161A1 I >

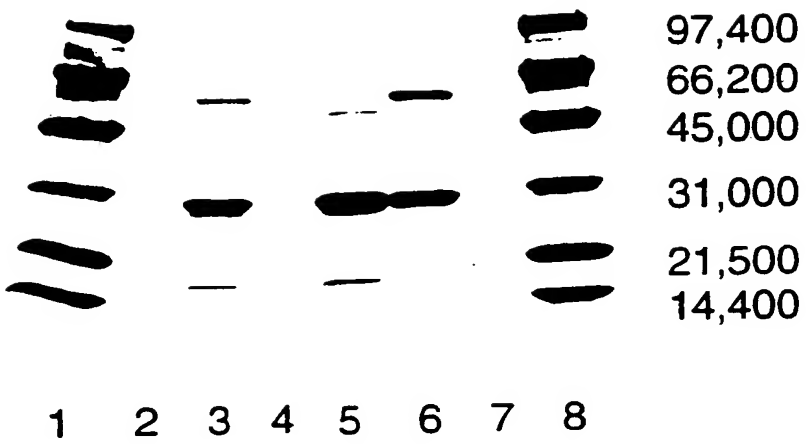


FIG. 26

DOCID <WO 9311161A1 I >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09965

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/28, 3/20; C07H 21/04; C12P 21/08; C12N 15/00, 15/03;
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3, 413; 435/7.92, 7.93, 7.94, 69.6, 69.7, 70.21, 172.2, 172.3, 240.27, 252.3, 320.1; 424/1.1, 85.8; 536/23.4, 23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG (FILES 5, 73, 155, 351); U.S. AUTOMATED PATENT SYSTEM (FILE USPAT, 1971-PRESENT).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 88/09344 (HUSTON et al.) 01 DECEMBER 1988, see entire document.	1-33,38-39,44-63 34-37,40-43
X Y	US, A, 4,946,778 (LADNER et al.) 07 AUGUST 1990, see entire document.	32-33,38-39 1-31,34-37,40-63
Y	CANCER RESEARCH, Vol. 48, issued 15 AUGUST 1988, Muraro et al., "Generation and Characterization of B72.3 Second Generation Monoclonal Antibodies Reactive with the Tumor-associated Glycoprotein 72 Antigen," pages 4588-4596, see entire document.	34-37,40-43
Y	SCIENCE, Vol. 242, issued 21 OCTOBER 1988, Bird et al., "Single-Chain Antigen-Binding Proteins," pages 423-426. See entire document.	32-42
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 265, No. 30, issued 25 OCTOBER 1990, Bedzyk et al., "Immunological and Structural Characterization of a High Affinity Anti-fluorescein Single-chain Antibody," pages 18615-18620. See entire document.	32-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
23 FEBRUARY 1993

Date of mailing of the international search report
05 MAR 1993

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
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Authorized officer
ROBERT D. BUDENS
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

DOCID <WO___9311161A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09965

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/387.3, 413; 435/7.92, 7.93, 7.94, 69.6, 69.7, 172.3, 252.3, 320.1; 424/1.1, 85.8; 536/23.53

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-14, 19-29, 31, 44-46, 51-58, a first product, method of making and method of using, drawn to multivalent antigen-binding proteins, compositions, methods of making multivalent proteins and method of using multivalent proteins to detect antigens, classified in Class 530, subclass 387.3 and Class 435, subclasses 7.1, 69.7, 172.3, 320.1, 252.3.

II. Claims 15-18, a second product, drawn to compositions containing multivalent proteins and single chain proteins, classified in Class 530, subclass 387.3.

III. Claim 30, a second method of use, directed to a method of imaging, classified in Class 424, subclass 85.8.

IV. Claims 32-37, a third product, drawn to single chain proteins classified in Class 530, subclass 387.3.

V. Claims 38-43 and 47-50, a fourth product, drawn to genetic sequences, vectors and hosts, classified in Class 536, subclass 23.53, Class 435, subclasses 320.1 and 252.3.

VI. Claims 59-61, a third method of use, drawn to immunoassay methods, classified in Class 435, subclasses 7.92, 7.93 and 7.94.

VII. Claim 62, a fourth method of use, drawn to a method of immunotherapy using multivalent proteins, classified in Class 424, subclass 85.8.

VIII. Claim 63, a fifth method of use, drawn to a method of immunoaffinity purification using multivalent proteins, classified in Class 530, subclass 413.

The inventions of Groups I-II and IV-V are directed to multiple products which differ in their physical properties such as primary sequence, molecular weights and chemical composition and are not so linked as to have a common special technical feature.

Further, the methods of Groups I, III and VI-VIII differ in their utilities, reagents and method steps and are not so linked as to have a common special technical feature.

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